A Study of Tumor Suppressor Genes in Multiple Myeloma

by

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Abstract

p16 and p15, both encoded by the genes located on chromosome 9p21, are inhibitors of cyclin-dependent kinases CDK4/6 and the up-stream regulators of *Rb* function. Alterations of *p16/p15* have been commonly described in hematopoietic malignancies, particularly lymphoid and B-lineage origin. However, the involvement of these two genes in multiple myeloma (MM) has not been reported. The aims of our current study are (1) To investigate whether these cell cycle-related genes (*Rb*, *p16* and *p15*) functioning in the G1-S transition are altered in MM and (2) To evaluate their potential roles in MM pathogenesis through the analysis of clinico-pathologic correlation.

By a combination of Southern blot hybridization and Polymerase Chain Reaction- Single Strand Conformation Polymorphism (PCR-SSCP) analysis, we observed, consistent with findings in the literature, no deletions or mutations of *p16* and *p15* and no homozygous deletions or gene rearrangements of *Rb* in our twenty-four MM patients analyzed. However, hypermethylation was observed in 67% for *p16* and 58% for *p15* in our group of relatively high stage MM patients. Such high frequencies of involvement of these genes in MM make them hitherto the most common genetic abnormalities in this disease. Although expression studies were not performed, our findings of reciprocal genetic alterations in *Rb* and *p16/p15* are in line with observations made on other solid tumors involving these genes.

A significant correlation (p=0.026) between the blastic disease and hypermethylation of *p16* gene was also demonstrated. The fact that hypermethylation of *p16/p15* was found in both low and high stage patients and in similar incidences in pre- and post-treated groups may suggest they are early events and their role in tumor initiation can be speculated. Concomitant hypermethylation of

p16 and *p15*, uncommon thus far in the literature of the study of these genes, is a rather common phenomenon, occurring in 54% of our patients. Since all three patients (100%) having plasmacytomas were found to have concomitant hypermethylation of both *p16* and *p15* genes while this would only be seen in 48% of the non-plasmacytoma group (n=21), this may suggest that concomitant hypermethylation of both genes may be pathogenetically related to plasmacytoma development.

Our study is the first demonstration that alterations of *p16* and *p15* are involved in high incidences of MM, not by homozygous deletions or mutations, but solely by hypermethylation of 5'CpG islands which may be a specific mechanism in this disease. With the sensitive methylation-specific PCR (MS-PCR) technique developed recently, the methylation status in MM may serve as a tumor marker for the detection of minimal residual disease and evaluation of tumor contamination in the autologous graft in autologous bone marrow transplantation. Therapeutic innovations in drug development and processing of marrow or peripheral stem cell grafts resulted from information of this study may be speculative at this stage but effective in future.

多發性骨髓瘤的腫瘤抑制基因研究

摘　　要

P16和P15蛋白，被位於九號染色體短臂二區、一帶的基因編碼，是cyclin依賴性激酵素CDK4/6的抑制者和Rb功能的調節者。P16/P15的改變在血液惡性腫瘤中，特別是在淋巴及B細胞源性惡性腫瘤中已被廣泛描述。但是，在多發性骨髓瘤中，這兩個基因是否參與尚未報到。本研究的目的是：(1) 調查在多發性骨髓瘤中，這些在G1-S過渡期有功能的細胞周期相關性基因 (Rb，P16和P15) 的改變；(2) 通過臨床和病理關係的分析，評估他們在多發性骨髓瘤中的潛在角色。

通過Southern blot雜交和聚合酵素鏈式反應—單鏈構象多態性分析(PCR-SSCP)，我們觀察所得，與文獻記載的發現一致，就是在分析的24例多發性骨髓瘤中，沒有P16和P15 的缺失和突變，及Rb的同源重組。然而，在晚期的多發性骨髓瘤中，我們觀察到67%的P16和58%的P15存在高度甲基化。在多發性骨髓瘤中，這些基因如此高頻的參與，使它們成爲該病最普遍的遺傳改變。盡管未做表達性的研究，我們發現的Rb和P16/P15遺傳改變的相反關係模式，與這些基因在實體瘤中的表現相一致。

髓性疾病與P16基因的高度甲基化呈現高度的相關性(P=0.026)。P16/P15的高度甲基化在疾病的晚期和早期，治療前後的病人中均具有相似的頻率，可建議甲基化爲早期事件，並且推測其在腫瘤發生中起始動作用。P16和P15的共甲基化的情形在目前關於這些基因的研究中並不普遍，但在我們的病人中卻普遍存在，佔54%。因爲全部三個(100%) 伴有漿細胞瘤的病人均存有P16和P15共高度甲基化，而在非伴有漿細胞瘤組中(n=21)，僅有48%顯示共高度甲基化，這可建議兩個基因的共同高度甲基化可能和漿細胞瘤的病因學有關。

我們的研究首次揭示了P16和P15的高頻改變參與了多發性骨髓瘤的發生。這些改變，不是由缺失或突變造成，而單是由5'CpG島的高度甲基化所導致。此改變或許是該病發生的一個特定機制。利用近來發展之敏感的甲基化特異性聚合酵素鏈式反應(MS-PCR)技術，甲基化的狀態可以成爲檢查微小殘餘疾病和評價在骨髓移植中，自家移植物的腫瘤污染之標志物。在藥物開發，及處理骨髓或外周幹細胞移植物方面，本研究所提供的信息可導致新治療方法的發明，在現階段或許是推測，但於將來，可以實現。

List of Abbreviations

ALL	Acute lymphocytic leukemia
AML	Acute myelogenous leukemia
bp	base pair
BM	Bone marrow
BMPC	Bone marrow plasma cell
cdks	cyclin-dependent kinases
C	Cytosine
CSF	Colony - stimulating factor
DNA	deoxyribonucleic acid
FISH	Fluorescence-in-situ-hybridization
G	Guanine
Hb	Hemoglobin
IFNγ	Interferon γ
IgH	Immunoglobulin heavy chain
IL	Interleukin
IPTG	Isopropyl-β-thiogalacto-pyranoside
LB	Luria-Bertani medium
MGG	May-Grunwald-Giemsa
MM	Multiple myeloma
MTS	Multi-tumor suppressor
PCR	Polymerase chain reaction

PLT	platelet
Rb	Retinoblastoma
SDS	Sodium lauryl sulfate
SSC	Sodium citrate and sodium chloride
SSCP	Single strand conformation polymorphism
TGF	Transforming growth factors
Tx	Treatment
VHL	von hippel-lindau gene
WBC	White blood cell
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
5-mcyt	5-methylcytosine

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Chapter 1

Introduction

1.1 Multiple Myeloma

Multiple myeloma (MM) is a relatively rare clonal B-cell neoplasm characterized by skeletal dissemination of malignant plasma cells that accumulate in bone marrow and secrete monoclonal immunoglobulin (Ig), the paraprotein. The skeletal involvement is mainly in axial skeleton, manifested as osteolytic lesions in skull, vertebrae and pelvic bones that cause pain and pathological fractures. Patients with early stages of MM may be asymptomatic and anaemia, infections, bone pain or fractures, renal failures or rarely bleeding tendency may develop at later stages and are the first presenting features at diagnosis. The homogeneous monoclonal immunoglobulins or Ig fragments, known collectively as M components detected by immuno-electrophoresis, are composed of a single heavy chain class and a single light chain class. Ninety-nine percent of patients have either in their serum or urine a monoclonal M protein and the rest have non-secretary type of myeloma. According to the data in the West (Postgraduate hematology, Hoffbrand et al, 1989), IgG (55.7%) is the most common M protein, followed by IgA (26.7%), solely light chain (14.7%), IgD (1.5%) and IgM (0.2%). κ-light chain is more common than λ-light chain. Very rarely, biclonal M protein is seen. Many patients have low levels of other normal Igs. This immunoparesis increases the susceptibility of patients to infection, which is one of the leading causes of death among myeloma patients.

1.2 The Problem

As a result of the low mitotic yield and also often complex karyotypic changes, specific cytogenetic abnormality in MM has not been found (Dewald et al,1985; Barlogie et al,1989; Ferti et al, 1984; Gould et al, 1988). Unlike most other hematological malignancies, no consistent specific translocation or structural anomalies have been reported. This fact has hindered the guided approach of molecular genetic studies that are commonly employed in leukemias and may otherwise yield more relevant results in this malignancy. A normal karyotype was seen in 50-60% of MM (Riedel et al, 1992). However, this needs to be interpreted with great caution as it was observed by flow cytometry that a high frequency (>80%) of DNA aneuploidy actually existed (National Cancer Institute, Annual Cancer Statistics Review, 1992). Nowadays, results from fluorescence in-situ hybridization (FISH) studies have suggested that almost all MM patients are cytogenetically abnormal (Zandecki et al, 1996)

On the other hand, molecular genetic data of MM are not systematic. No consistent oncogene or tumor suppressor gene abnormalities have been found in human myeloma while variable abnormalities of expression in c-*myc*, *ras*, *Rb* and *p53* have been described. c-*myc*, *bcl-1* and *bcl-2* involved in inhibition of apoptosis are highly expressed but no gene rearrangements have been detected thus far (Van den Berghe et al, 1989; Durie et al, 1992; Barlogie et al, 1995). Despite frequent increased *c-myc* and *bcl-2* expression (Pettersson et al, 1992; Selvanayagam et al, 1988; Ong et al, 1995) and the rather frequent occurrence of t(11;14) (q13;q32) involving the *bcl*-1 (Pettersson et al, 1992; Ladanyi et al,

1992), structural rearrangements of these genes are rare (Niesvizky et al, 1993; Ladanyi et al, 1992).

Essentially a disease of the elderly, MM has a peak incidence in individuals at 70 to 80 year of age. The age adjusted incidence rates in the Chinese of Hong Kong per 100,000 are 1.7 for men and 1.5 for women (Hong Kong Hospital Authority, Annual statistical report of Hong Kong, 1993), which are 2-3x lower than in the West. Data on the study of MM in Chinese are correspondingly fewer. Although the median age at diagnosis is 70 years, it is not uncommon for the disease to be diagnosed in much younger patients (< 40 y.o.). Moreover, there is a trend that younger patients (<30 y.o.) are also affected in recent years. Unfortunately, little improvement in the 5-year survival rates has been made since the mid-1970s despite advances in prognostication and the use of multiagent chemotherapy. The slow progress in terms of treatment strategy compared to other hematological malignancies may be a reflection of our poor understanding of MM pathogenesis. To date, this malignancy is still regarded as a uniformly fatal disease not curable by chemotherapy. Although hereditary factors, radiation exposure, agricultural exposure, and antigenic stimulation have all been implicated to some extent, the etiology and pathogenesis of MM remain obscure.

Chapter 2

Literature Review

2.1 Molecular genetics of multiple myeloma

2.1.1 Cytogenetics

The most frequent abnormalities described so far in multiple myeloma(MM) are listed in Table 1. An abnormal karyotype is found in approximately 50% of patients, while the use of FISH and DNA content of plasma cells demonstrates abnormalities in nearly 90% of the MM patients. The abnormal karyotypes generally show numerous numeric and structural aberrations, and some patients have multiple abnormal clones. However, although cytogenetic studies have been carried out in many patients with different types of plasma cell disorders, it is clear that no single pattern occurs in MM (Zandecki, 1996).

Most of the structural rearrangements identified so far have been those associated with B-cell neoplasms (Zandecki, 1996). 14q32 translocation is a non-random structural abnormality in MM, occurring in about 20-30% of cases with an abnormal karyotype. The most frequent 14q32 translocation is t(11;14)(q13;q32.3), occurring in about 10-25% cases with abnormal karyotypes (Zandecki, 1996) Many other translocations have also been reported, including t(8;14)(q24.1;q32.3),

t(14;18)(q32.3;q21.3) and t(6;14)(p21.1;q32.3) (Gould et al, 1988; Nishida et al, 1989a,b).

Table 1. Main numeric and structural changes that have been reported in multiple myeloma.

Numeric changes	
Gains: 9, 3, 19, 15, 11, 7, 5, 18, 21 (trisomy or tetrasomy)	
Losses: 13*, X (females), 14, 8, Y (monosomy or nullosomy)	
Structural changes	Main associated changes
14q+	(11;14)(q13;q32) (8;14)(q24;q32) (14;18)(q32;q21) (6;14)(p21;q32)
16p or 16q	(1;16)(p11;p11) (1;16)(q10;p10)
1p or 1q (partial deletion, trisomy 1q)	
19q13 or 19p13	
6q	
17q	
2p12 or 22q11	
7q	
	(9;22)(q34;q11)

* Loss of whole or part of chromosome 13.

Adapted from Zandecki, 1996

6q abnormalities have been found in approximately 10% of MM patients. This abnormality is associated with increased levels of osteoblast activating factor (OAF) and TNF production and may imply increased risk of osteolytic bony involvement (Gould et al, 1988).

7q abnormalities are also relatively common. Such deletions are associated with the expression of the multi-drug resistance (MDR) phenotype since this is the locus of the gene encoding for p glycoprotein, product of the mdr-1 gene. Over-expression of membrane p glycoprotein may lead to activation of an ATP-dependent efflux pump which expels drugs from the cell (Dalton et al, 1992).

Structural abnormalities of chromosome 1 have been described in about 40-50% of MM patients and abnormal karyotypes involving translocations, deletions, duplications and inversions (Zandecki et al, 1996)

Two structural aberrations, both involving 16p, were reported in MM. Flactif et al (1994) reported a t(1;16)(p11;p11) in two patients, and chromosome painting confirmed the abnormality as located at 16p11.

Structural aberrations for other chromosomes have also been described. Those involving chromosome 19 have been reported by two studies (Taniwaki et al, 1994; Laï et al, 1995). Laï et al (1995) described 19q13 abnormality in 12.5% of patients with an abnormal karyotype. Taniwaki et al (1994) found that a breakpoint at 19p13.3 was recurrently involved in translocations of chromosome 19.

Fluorescence *in situ* hybridization (FISH) has enabled detection of numeric chromosome changes in both interphase and metaphase cells using DNA probes directed against one part of a chromosome (Hasegeli Uner et al, 1994). FISH techniques can be performed on bone marrow smears, cytospins, and cytogenetic preparations (Lee et al, 1993; Flactif et al, 1995). By interphase FISH analysis using probes for 5 and 10 different chromosomes, respectively, two studies reported that at least one chromosome is trisomic in 96% or 89% of myeloma (Drach et al, 1995; Flactif et al, 1995). The significance of the numeric chromosomal abnormalities is unclear in MM; they may be associated with tumor progression whereas structural aberrance may be more important in the origin of the clonal neoplastic disorder (Dewald & Jenkins, 1991).

The low mitotic yield and complexity of karyotypic changes both have been a Hindrance to a directed approach to the study of oncogenes and suppressor genes (Zandecki, 1996). A number of genes recognized to be important in other B-cell malignancies were found to abnormal in MM namely, *c-myc, bcl*-1, *bcl*-2, N-*ras,* and H-*ras*. Abnormalities of *Rb, p53* and other tumor suppressor genes were also described. Studies at the molecular level have revealed alterations in proto-oncogenes, tumor-suppressor genes, and most recently, cell-survival genes in patients with multiple myeloma.

2. 2 Alterations of proto-oncogenes

2. 2. 1 *c-myc*

The myc proto-oncogenes encode nuclear phosphoproteins involved in the control of cellular proliferation and differentiation (Feinman et al, 1997). The *c-myc* gene (on chromosome 8q24) plays a role in the pathogenesis of a number of B-cell malignancies (Varterasia, 1995). However, in myeloma, gross structural changes involving chromosome 8 are rarely seen (Varterasia, 1995). Deregulation of *c-myc* appears to participate in the pathogenesis of MM since high level of *c-myc* expression has been seen. The elevated *c-myc* expression might be caused by mutation in the 5'-untranslated region of the *c-myc* gene leading to a deregulated translational control of c-myc gene expression (Paulin et al, 1996).

2. 2. 2 Ras

The p21/*ras* signaling transduction pathway integrates a variety of growth and inhibitory signals that are critical for cellular proliferation and differentiation. In a recent study, a high incidence of *ras* mutations (49%), with N-*ras* being the most frequent, was detected in 160 newly diagnosed MM patients (Liu et al, 1996). A significant correlation with tumor burden and survival was seen only in those patients harboring a K-*ras* mutation.

2. 2. 3 BCl-2 and related proteins

Expression of bcl-2 is thought to play an important role in B-cell malignancies. In myeloma, the t(14;18) translocation, typical for follicular lymphoma which results in over-expression of bcl-2, occurs at low frequency (2-3%) (Sawyer et al, 1995). However, over-expression of bcl-2 is seen in the majority of MM and MM cell lines (Ong et al, 1995; Pettersson et al, 1992; Ladanyi et al, 1992). Hypomethylation was proposed as a potential mechanism for the upregulation of the bcl-2 expression (Hanada et al, 1993). Over-expression of bcl-2 in cancer cells can result in chemoresistance and blocks apoptosis (Hallek et al, 1998).

2. 3 Alterations tumor-suppressor genes

2. 3. 1 *p53* gene mutations

The *p53* tumor-suppressor gene encodes a nuclear DNA-binding protein that is active in cell-cycle regulation (Varterasian, 1995). The *p53* gene has many effects on cell growth and differentiation and is often viewed as a gate-keeper to enter the cell cycle (Hallek et al, 1998)..

p53 mutations are very rare in MM, and appear to be a late event in the disease (Yasuga et al, 1995) . *p53* mutations occur only in about 5% of inactive MM (Portier et al, 1992).

2. 3. 2 Retinoblastoma (*Rb*) gene

The retinoblastoma tumor suppressor gene product (pRb), a nuclear phospoprotein that suppresses the G1-S in the cell cycle, plays an important role in cell growth and differentiation. (Weinberg, 1995; Livingston et al, 1989; DeCaprio et al, 1989; Adams et al, 1996; Weinberg, 1991; Weinberg, 1993). pRb function is regulated by phosphorylation. Dephosphorylated pRb is active form and that prevents the cell to enter the S phase and hence induces the cell-cycle arrest; in contrast, pRb is phosphorylated and hence inactivated by the cyclin dependent kinase (CDK) CDK4/6-cyclin D complex thereby promotes the entry of cells into S phase (Weinberg, 1995). Alterations of the *Rb* gene or abnormalities in pRb have been described in up to 70% of MM patients and 80% of MM-derived cell lines (Corradini et al, 1994; Dao et al, 1994; Juge-Morineau et al, 1995). By Southern blot or interphase FISH analysis, monoallelic deletion of *Rb* (or loss of one copy of chromosome 13) has been reported in about 50% to 60% of primary MM and cell lines respectively, independent of the disease stage. Bi-allelic deletion of *Rb* was found in 1 of 22 MM cell lines and in 1 of 10 primary tumors (Corradini et al, 1994; Dao et al, 1994; Juge-Morineau et al, 1995). The monoallelic lesions were not associated with the pRb expression, and no mutations or rearrangements of *Rb* have been described (Juge-Morineau et al, 1995). These findings suggest that a bi-alleilic loss of the *Rb* gene is infrequent in MM and thus inactivation of *Rb* appears to be only a rare oncogenic event in MM. Since pRb can suppress IL-6 (Santhanam et al, 1991) which is the

crucial growth factors of MM, the factors involves in the upstream control of the *Rb* function and cyclin D-Rb pathway still worth further investigation.

2. 3. 3 *p16* and *p15* genes

A major candidate for the chromosome 9p21 tumor suppressor gene is *p16* (Kamb et al, 1994; Nobori et al, 1994). Later on, another tumor suppressor gene *p15* has been identified on 9p21 near the *p16*. Both *p16* and p15 are the inhibitors of cyclin dependent kinases CDK4/6 by competing with cyclin D for binding to CDK4/6 and hence preventing the formation of CDK-cyclin D complex which can phosphorylate the pRb (Kato et al, 1993). Deletions or mutations in the *p16* or *p15* gene may affect the balance between functional *p16*/*p15* and cyclin D, resulting in abnormal cell cycling and growth. Mutations of *p16* were described in melanoma cell lines (Kamb et al, 1994; Nobori et al, 1994) and demonstrated in the germ line of several melanoma kindreds linked to 9p21 (Hussussian et al, 1994; Kamb et al, 1994). Multiple small studies showed that no homozygous deletions or mutations of *p16*/*p15* genes are involved in MM (Quesnel et al, 1995; Hangaishi et al, 1996; Ogawa et al, 1995).

Recently, inactivation of *p16*/*p15* by an alternative mechanism, *de novo* methylation of 5'CpG island, has been demonstrated in many tumors (Merlo et al, 1995; Herman et al, 1995; Gonzalez-Zulueta et al, 1995; Lo et al, 1996). Such a mechanism, which has been a focus of attention in the study of oncogenesis, has not been examined in MM.

Chapter 3

DNA methylation and Cancers

3.1 Role of DNA methylation

It is getting clear that DNA methylation, essential for normal embryonic development, plays an important role in carcinogenesis. Alterations in DNA methylation are very common in cancer cells. Current interest in the role of methylation has focused on the potential of aberrant methylation in silencing tumor suppressor genes, a novel pathway on the development of progressive epigenetic inactivation. Random methylation errors resulting in the *de novo* methylation of CpG islands on the promoter region may contribute to the progressive inactivation of growth-inhibitory genes resulting in the clonal selection of cells with growth advantage. Promoters silenced by methylation can be reactivated by treatment with the demethylating agent 5-aza-2'deoxycytidine. This mechanism of gene inactivation in carcinogenesis has important clinical implications, since it is possible to reactivate these dormant genes using inhibitors of DNA methylation and potentially restore growth control of cells (Jones, 1996).

3.2 CpG islands

CpG islands are G+C rich regions that show a high frequency of CpG dinucleotides and usually unmethylated in normal somatic cells (Bird, 1986), except during X-chromosome inactivation (Singer-Sam et al, 1993), Alu and L1 sequences (Liu and Schmid, 1993) and parental specific imprinting (Li et al,

1993). However, widespread methylation of the CpG islands was observed on autosomal genes during oncogenic transformation (Jones and Buckley, 1990; Laird and Jaenish, 1994). Many studies have demonstrated that aberrant hypermethylation is associated with inactivation of tumor suppressor genes.

3.3 Abnormalities of DNA methylation in neoplasia

DNA methylation changes are the most common detectable abnormalities in human neoplasia (Issa et al, 1997). There are three major alterations in the methylation system being described: (1) widespread loss of DNA methylation from normally methylated sites; (2) increased total activity of the DNA methyltransferase (Mtase); and (3) regional increases in DNA methylation, often involving the CpG islands in the promoters of selected genes such as the tumor suppressor genes Issa et al, 1997). Each of these changes has potentially profound consequences for DNA function and structure, and the interplay between them may be particularly important for neoplastic progression (Baylin et al, 1998).

3.3.1 DNA Hypomethylation in cancer

The earliest alteration of DNA methylation to be described in human neoplasia was the loss of 5-methyl-cytosine (Feinberg et al, 1983). This demethylathylation process has been proposed to play a role in potential

activation of growth promoting genes such as oncogenes and hence possibly the induction of chromosomal instability (Jones and Buckley, 1990). The decreased DNA methylation has been described in some oncogenes in human tumors, however, the demethylation is not always associated with reactivation at the gene expression level (Kochanek et al, 1991).

3.3.2 DNA methyltransferase activity in cancer

In addition to DNA hypomethylation, elevation in methylatransferase (Mtase) activity has also been observed in a variety of cancer cell lines and primary tumors (Kautiainen et al , 1986; Issa et al, 1993; Belinsky et al, 1996). Over-expression of Mtase may be important in pathogenesis of neoplasia since high level of Mtase activity results in a marked increase in overall DNA methylaltion which is accompanied by tumorigenic transformation in non-tumorigenic line NIH-3T3 (Wu et al., 1993). Another study demonstrated that phenotype in a malignant cell line could be reverted by inhibition of Mtase activity (MacLeod and Szyf, 1995). Their results support the hypothesis that the DNA Mtase is actively involved in oncogenic transformation. All these observations are consistent with a role for increased Mtase levels in neoplasia (Issa et al, 1997).

3.4 Regional DNA hypermethylation in cancer

Work during past 10 years on DNA methylation suggests that the normally unmethylated CpG islands in the 5' regulatory regions of genes would be a prime target for aberrant hypermethylation in tumor cells and is the most profound association between altered DNA methylation and changes in gene expression in neoplasia (Baylin et al, 1998). Dense methylation within a 5' regulatory region has the potential to participate in chromatin events that result in transcriptional silencing of an involved gene. Examples of such genes include the two cell-cycle regulators *p16* (Gonzalez-Zulueta et al, 1995) and *p15* (Herman et al, 1996), the von Hippel-Lindau gene (*VHL*) in some renal carcinomas (Herman et al, 1994), the retinoblastoma gene *Rb* (Stirzaker et al, 1997), the *BRCA* 1 (Dobrovic and Simpfendorfer, 1997) and the metastasis-suppressor gene-cadherin (Graff et al, 1995).

3.4.1 *p16* and *p15* genes in solid tumors

The function and the role in cyclin D-Rb pathway of the two cell-cycle regulators p16 and p15 have been addressed previously (see Chapter 4 Background of studies). In many human cancers, the common structural lesion of chromosome 9p21 is homozygous deletion of an region residing not only the *p16* gene but also the nearby *p15* gene (Jen et al, 1994; Kamb et al, 1994). However, studies of methylation patterns for the *p15* and *p16* genes in solid tumors have pointed to the more important role for the *p16* gene (Baylin et al,

1998). Among most solid tumor types studied, the frequency of hypermethylation of a 5' CpG island of the *p16* gene in primary tumors ranges from 20 to 67% (Baylin et al, 1998). Only the *p16* gene is frequently methylated when copies of both *p16/p15* genes are retained in solid tumors (Herman et al, 1995, 1996) with the exception that the *p15* gene methylation is seen in lung cancer cell lines, and almost always with concomitant hypermethylation of the *p16* gene (Herman et al, 1996). Also, although point mutations are not frequent in the *p16* gene, they do occur, but these have not been documented in the *p15* gene (Herman et al, 1995).

3.4.2 The *p16* and *p15* genes in leukemia and other hematopoietic malignancies

In tumors of hematopoietic origin, the patterns and mechanisms of inactivation for the *p16* and *p15* genes differ from those in solid tumors. The loss of *p15* gene function is a dominant event in tumors derived from the hematopoietic cell system, and CpG island hypermethylation is by far the dominant mechanism involved (Baylin et al, 1998). Large studies of patients with both the adult and pediatric types of acute myelogenous leukemia (AML) showed hypermethylation on *p15* in 86% and 65% respectively; (Herman et al, 1996; Herman et al, 1997). No homozygous deletions or mutations of either the *p15* or *p16* genes have been involved, and the *p16* gene is not concomitantly hypermethylated (Herman et al, 1996; Herman et al, 1997). The methylation

pattern seen for AML also observed in adult acute lymphocytic leukemia (ALL) and pediatric B lineage subtype (Baylin et al, 1998). The high frequency of the involvement of *p15* may be due to that normal hematopoietic stem cells are sensitive to TGF-β-mediated growth inhibition response (Baylin et al, 1998).

For the lymphomas, *p15* and *p16* are rarely involved in low-grade non-Hodgkin's lymphoma (NHL), and hypermethylation of the *p16* gene alone was the only molecular event observed. In contrast, more than 80% of high grade NHL have hypermethylation of the *p16* gene (Baylin et al, 1998). In Burkitt's lymphoma, both the *p15* and *p16* gene are frequently simultaneously hypermethylated, and methylation of one gene or the other is a common phenomenon in this neoplasm.

Studies of the *p16* and *p15* genes on chronic myelogenous leukemia (CML) showed that no involvement of either the *p16* or *p15* genes by mutation or hypermethylation in any stage of CML (Herman et al, 1997).

3.4.3 Retinoblastoma gene

The *Rb* gene was the first classic tumor suppressor gene in which CpG island hypermethylation was detected. The change has been noted in tumor DNA from about 10% of patients with the non-genetic or sporadic form of retinoblastoma (Sakai et al, 1991). Although the *Rb* gene is commonly mutated in many forms of human cancer, promoter region hypermethylation has been reported only in retinoblastomas (Baylin et al, 1998).

3.5 Mechanism underlying the DNA methylation changes

In malignant cells, the 5' CpG-island regions become methylated and the associated gene is transcriptional silencing (Counts and Goodman, 1995). In the case of growth-regulatory genes such as the tumor-suppressor gene, this may result in clonal selection of cells with growth advantage. DNA methylation-mediated transcriptional inhibition has thus been proposed as alternative pathway to the inactivation of tumor suppressor genes other than mutation and deletion (Jones,1996).

A recent study by Chuang et al (1997) has proposed new pathways for the formation of methylation errors and mutations in cancer cells related to the proliferating cell nuclear antigen (PCNA) which is an auxiliary factor for DNA replication and repair. It was shown that the DNA methyltransferase (Mtase) methylates the newly replicated DNA by binding to the replication associated protein PCNA. The cell cycle regulator $p21^{WAF1}$ can disrupt the DNA Mtase interaction which suggests that 21 may regulate methylation by blocking access of Mtase to PCNA. DNA Mtase and p21 may be linked in a regulatory pathway since the DNA Mtase is competing with p21 for PCNA and the extents of their expression are inversely related in both normal and SV40 transformed cells (Chuang et al, 1997; Waga et al, 1994).

In a malignant cell, the loss of p21 would perturb a balance between the p21 and DNA-methyltransferase interaction with PCNA, which would allow for an

increase in methylation errors (Robertson and Jones, 1997). On the other hand, the loss of p21 may also allow the methyltransferase access to sites of DNA damage, resulting either in a methylation error or in *de novo* methylation at a 5'CpG site that is normally unmethylated. This type of random methylation error will then be potentially leading to the inactivation of the tumor suppressor genes (Robertson and Jones, 1997).

Chapter 4

Background of study

4.1 Background of study

Although many studies have been conducted in the investigation of MM pathogenesis in recent years, there are still many gaps of information with unresolved issues in this uniformly fatal cancer. The finding of a high incidence (>50%) of *Rb* deletions in MM by Dao et al in 1994 has started a new line of approach in the study of molecular genetics, providing insight for further research in this malignancy (Dao et al, 1994). The potential involvement of this classical tumor suppressor gene in MM pathogenesis may have major implications because 1. *Rb* plays a critical role in the control of cell cycle progression by suppressing cell growth at the G1 phase. Loss of Rb function would lead to cellular proliferation and transformation; and 2. More importantly, *Rb* gene product (pRb) suppresses the gene transcription and hence production of interleukin-6 (IL-6) (Santhanam et al, 1991), which is a crucial myeloma growth factor that also prevents cell apoptosis (Lichernstein et al, 1995). *Rb* deletions may result in deregulation of IL-6 expression and hence inhibition of apoptosis and expansion of IL-6 dependent myeloma clone. However, later study by Juge-Morineau et al demonstrated that despite the frequent *Rb* deletions (81% in human myeloma cell lines (HMCL) and 70% in MM patients) being found in MM, a majority of the *Rb* deletion were hemizygous and not associated with the loss of expression of the pRb. Bi-allelic deletions with loss of pRb expression were rare (4.5% in HMCL and 10% in MM patients).

As a negative regulator of cell cycle, *Rb* is subjected to the control of a broad array of cyclins and cyclin-dependent inhibitors (CDIs) that play critical roles in tumorigenesis by governing balance of cell growth, division, survival and death (Karp et al, 1995). As illustrated in Diagram 1, *p16* and *p15* participate in the upstream regulation of *Rb* function by modulating the interaction of cyclin D and the cyclin-dependent kinase 4 (CDK4)/CDK6, the complexes of which can phosphorylate and hence inactivate the pRb (Kato et al, 1993). Phosphorylation of pRb and related G1 to S phase transition is triggered by activation of cyclin D-CDK4/CDK6 complex (Hirama et al, 1995). As cyclin-dependent kinase inhibitors, p16 and p15 compete with cyclin D for binding to CDK4/CDK6, resulting in dephosphorylation (hence activation) of pRb and related G1 growth arrest (Hirama et al, 1995; Serrano et al, 1993).

Encoding respectively a 16-KD and 15-KD protein, *p16* and *p15*, close neighbors both mapped to chromosome 9p21 region, are tumor suppressors in multiple human neoplasias (Okuda et al, 1995; Ogawa et al, 1995; Quesnel et al, 1995; Stranks et al, 1995; Merlo et al, 1995; Herman et al, 1995). The unique feature of *p16* is that inactivation is mainly by homozygous deletions in many primary tumors and co-deletion with *p15* is common (Ogawa et al, 1995; Quesnel et al, 1995). In addition, *p15* is up-regulated by TGF-β, which is a potent growth suppressor of hematopoietic progenitors in the bone marrow environment where a majority of myeloma cells are accumulated.

If *Rb* is not the major target gene in MM pathogenesis, as suggested by previous data of rare *Rb* inactivation and the associated loss of pRb expression,

abnormalities (e.g. *p16* and *p15*) in the upstream control of *Rb* function deserve further investigation. It has been demonstrated that MM cells may show a very strong expression of pRb (Zukerberg et al, 1996) that is mostly in its phosphorylated (inactivated) form (Urashima et al, 1996). However, homozygous deletions or point mutations in the coding regions of p16 and p15 were not observed in multiple small studies on MM (Ogawa et al, 1995; Quesnel et al, 1995; Hangaishi et al, 1996).

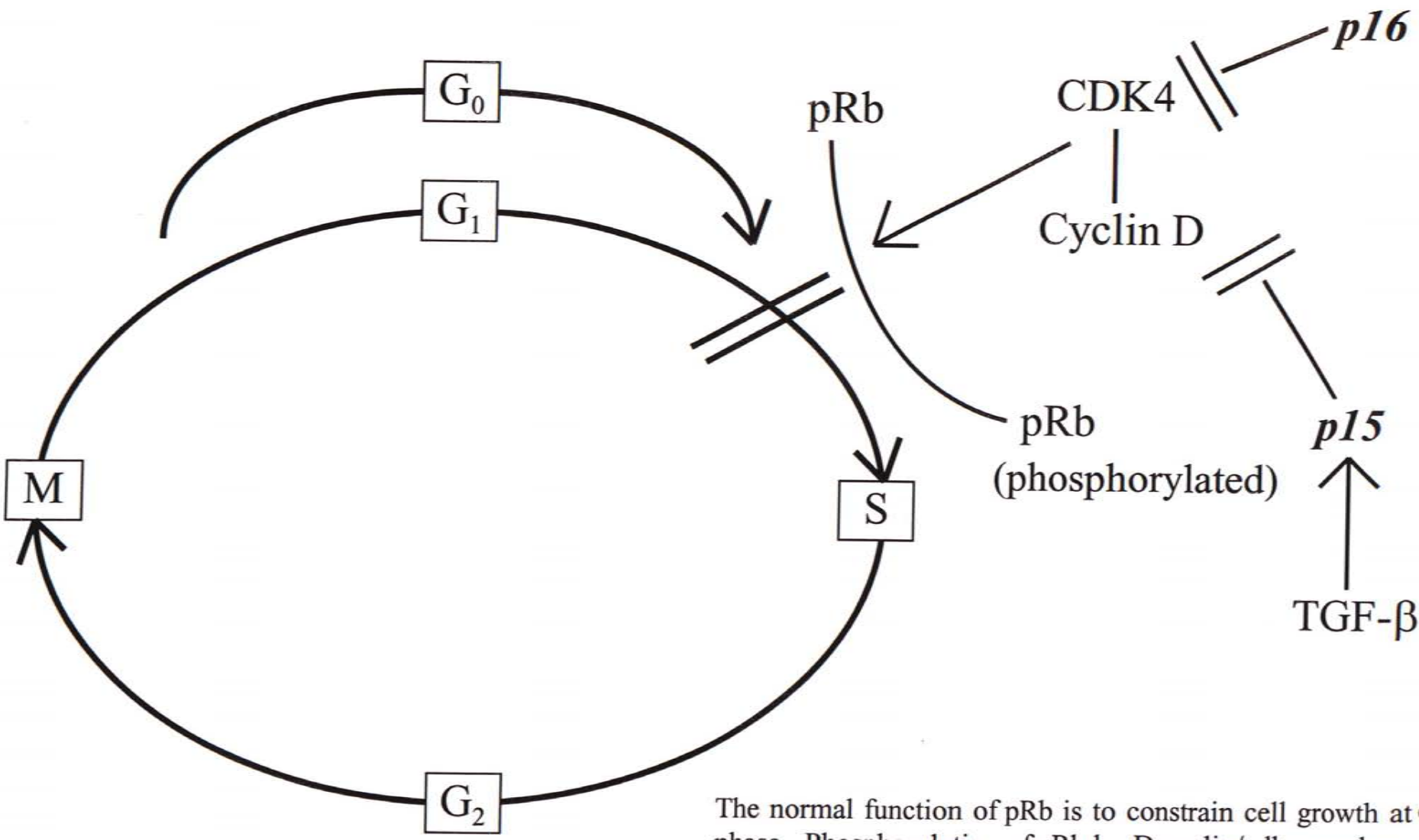
Recently, *de novo* 5'CpG island hypermethylation has been found to be associated with transcriptional silencing of *p16/p15* in many human cancers with and without previously observed homozygous deletions (Merlo et al, 1995; Herman et al, 1995; Gonzalez-Zulueta et al, 1995; Lo et al, 1996). These findings have more strongly affirmed *p16* as the most commonly involved tumor suppressor gene in human neoplasias. It also adds more weight to the tumor suppressor role of *p15* which, in acute myeloid leukemia (AML), has been shown to be predominantly involved through this aberrant methylation (Okuda et al, 1995).

Thus far, studies on the methylation status of *p16* and *p15* genes in MM have not been reported. It would be important to examine whether these two genes could be inactivated by this alternative mechanism of gene inactivation in MM-hypermethylation.

4.2 Project objectives

The current study aims to investigate whether the *p16/p15* genes are inactivated by deletions, mutations or hypermethylation of the 5'CpG islands that may serve as an alternative mechanism of inactivation of these genes in MM. The relationship between the status of *Rb* and *p16/p15* will also be examined by Southern blot analysis. Finally, a clinico-pathological correlation will be performed to evaluate the potential roles of *p16/p15* in MM pathogenesis and presence of potential biological subtypes, which if found, may alter treatment strategy and outcome.

Diagram. 1 **Genetic Control of Cell Cycle**



The normal function of pRb is to constrain cell growth at G_1 phase. Phosphorylation of pRb by D-cyclin/cdk complex will inactivate pRb and hence relieve the cell from pRb's growth suppression. Inhibition of cdk4/6, interaction with D1-cyclin by *p16/p15* may render them unable to phosphorylate hence inactivate pRb.

Chapter 5

Materials and Methods

5. 1 Patient samples

Bone marrow materials from twenty-four patients diagnosed of multiple myeloma in Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong, between May 1995 to March 1996 were recruited for the study. The diagnosis and staging classification followed the criteria by Durie and Salman (1975)(Table 2). Morphologically, the multiple myelomas were classified into three histological subtypes, namely, a. mature (>80% of myeloma cells had mature cellular morphology), b. intermediate (mixture of mature and immature myeloma cells present, of which 50-80% had mature morphology) and c. blastic (>50% of myeloma cells were blastic with nucleolations and dissociation of nuclear and cytoplasmic maturation) through the assessment on the May-Grunwald-Giemsa stained bone marrow aspirate smears and H & E sections of marrow clot and trephine biopsies.

5. 2 Normal controls

Twelve normal peripheral blood samples were obtained from the spouses of patients with thalassaemia traits as part of their premarital check. The spouses were healthy and with normal complete blood pictures. A bone marrow aspirate sample was obtained from a healthy marrow donor for allogeneic bone marrow transplantation.

Table 2 Criteria for diagnosis of multiple myeloma

Major criteria

I. Plasmacytoma on tissue biopsy
II. Bone marrow plasmacytosis with > 30 % plasma cells
III. Mococlonal globulin spike on serum electrophoresis exceeding 3.5 g /dl for IgG peaks or 2.0 g for IgA
peaks, ≥ 1.0g /24h of κ or λ light chain excretion on urine electrophoresis in the absence of amyloidosis

Minor criteria

a. Bone marrow plasmacytosis with 10 % to 30 % plasma cells
b. Monoclonal globulin spike present, but less than the levels defined above
c. Lytic bone lesions.
d. Normal IgM < 50 mg, IgA < 100 mg, or IgG < 600 mg / dl

Diagnosis will be confirmed when any of the following features are documented in symptomatic patients with clearly progressive disease.

The diagnosis of myeloma requires a minimum of one major + one minor criterion of three minor criteria that must include a+b.

1. I + b, I + c, I + d (I + a not sufficient)
2. II + b, II + c, II + d
3. III + a, III + c, III + d
4. a + b + c, a + b + d

Adapted from Durie and Salmon, 1975

5. 3 Storage of the samples

Samples were stored in -70°C bone marrow aspirates and normal peripheral blood samples either preserved in EDTA or heparin 0°C freezer until ready for DNA extraction.

5.4 Materials

5. 4. 1 Chemicals

Ultrapure dNTP set 2'-deoxynucleosides 5'-triphosphate : dATP, dCTP, dGTP and dTTP were purchased from Perkin Elmer/Cetus (Norwalk, CT USA). Acrylamide, N'N' -methylenebisacrylamide solution (2%) and acrylamide : N,N'-methylenebisacrylamide (29:1, 40%) were purchased from Amresco (Solon, Ohio,USA), Ammonium persulfate, and TEMED (N, N, N', N'-tetramethylethylenediamine) were purchased from Pharmacia Biotech (Uppsala, Sweden). Urea (Ultra-pure), Tris-base, SDS (Sodium dodecyl sulfate), EDTA (Ethylenediamine-Tetraacetic Acid), X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside), IPTG (Isopropyl-β-thiogalactopyranoside), Agarose and Ampicillin were purchased from Sigma Chemical Company (St. Louis, USA). [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000Ci/mmol) were purchased from Amersham Life Science (Amersham, U.K.). All other chemicals were purchased

from Sigma Chemical Company (St. Louis, USA) if not specified.

5. 4. 2 Primers

Primers used in the research, including primers for β-globin gene primers and primers for sequencing listed in Table 3 were purchased from Oligos Etc. Inc.(USA) and Gilco/BRL Life Technology (Gaithersburg, MD,USA).

Table 3 Primer sequences for analysis of *p16* and *p15*

Name	Sequences		References
M1-2F	5'-GAAGAAAGAGGAGGGGCTG-3'	*p16*, exon 1 (probe, SSCP)	Kamb *et al* , 1994
M1-1108R	5'-GCGCTACCTGATTCCAATTC-3'	*p16*, exon 1 (probe, SSCP)	Kamb *et al* , 1994
M2-42F	5'-GGAAATTGGAAACTGGAAGC-3'	*p16*, exon 2 (SSCP)	Kamb *et al* , 1994
M2-551R	5'-TCTGAGCTTTGGAAGCTCT-3'	*p16*, exon 2 (SSCP)	Kamb *et al* , 1994
X3.90F	5'-CCGGTAGGGACGGCAAGAGA-3'	*p16*, exon 3 (SSCP)	Hussussian *et al*, 1994
530R	5'-CTGTAGGACCCTCGGTGACTGATGA-3'	*p16*, exon 3 (SSCP)	Hussussian *et al*, 1994
15 Ex 1-F	5'-GAGGATCCGGGCCGCTGCGCGTCT-3'	*p15*, exon 1 (SSCP)	Sill *et al*, 1996
15 Ex 1-R	5'-TAGGATCCAGCCCCGATCCGCCGA-3'	*p15*, exon 1 (SSCP)	Sill *et al*, 1996
p15-1F	5'-CCAGAAGCAATCCAGGCGCG-3'	*p15*, exon 1 (probe)	Jen *et al*, 1994
p15-1R	5'-AATGCACACCTCGCCAACG-3'	*p15*, exon 1 (probe)	Jen *et al*, 1994
M2-89F	5'-TGAGTTTAACCTGAAGGTGG-3'	*p15*, exon 2 (SSCP)	Kamb *et al* , 1994
M2-50R	5'-GGGTGGGAAATTGGGTAA-3'	*p15*, exon 2 (SSCP)	Kamb *et al* , 1994

5. 4. 3 Enzymes:

Restriction enzymes *EcoR*I, *Eag*I, *Hind*III, *Sac*II and *Sma*I were purchased from Amersham Life Science (Amersham, U.K.) and New England Biolabs (Beverly, MA, USA). *Taq* DNA polymerase was purchased from Gibco/BRL Life Technology (Gaithersbury, MD, USA). T4 polynucletide kinase was purchased from United State Biochemical (Cleveland, OH, USA).

5. 5 Methods

5. 5. 1 Cloning of *p16* and *p15* exon 1 probes for Southern analysis

5. 5. 1. 1 PCR amplification of *p16* and *p15* exon 1 probes from normal blood DNA

Amplification of *p16* and *p15* exon 1 DNA fragments from normal blood DNA was obtained by using the published primer sequences listed in Table 3. The PCR conditions used for the amplification were one cycle at 95°C (5 min); four cycles at 95°C (10 s) with annealing temperature (T_{ann})=64°C (10s) and 72°C (10s); four cycles with T_{ann}=62°C; four cycles with T_{ann}=60°C; four cycles with T_{ann}=58°C; thirty cycles with T_{ann}=56°C; followed by final elongation at 72°C for 5 minutes. After 10% polyacrylamide gel electrophoresis, the band containing the DNA fragment of interest was excised from the gel and transfer to

an eppendorf tube. Autoclaved double distilled water 50 μl was added, and the sample was incubated at 65^0C for 6 hours. After spinning at high speed in a microcentrifuge, the elutant 2 μl was collected for re-amplification of the DNA fragment with the corresponding sets of primers and using the same conditions as described above.

5. 5. 1. 2. Recovery and purification of *p16* and *p15* exon 1 DNA fragments

The re-amplified DNA fragment was again electrophoresed and the band interested was excised from agarose gel, then the DNA fragment was recovered and purified for later cloning or direct sequencing.

Agarose gel (less than 250 mg) containing the DNA band to be extracted was excised by using a clean razor blade. The slice was cut into several smaller pieces, minced and transferred to a 1.5 ml microcentrifuge tube. The DNA fragments were eluted at 37°C water bath over night. After spinning at high speed for 1 minute in a microcentrifuge, the supernatant was collected in a clean microcentrifuge tube. The eluted DNA was precipitated by adding 2.5X volume of absolute ethanol and one tenth volume of 3M sodium acetate pH5.2 with addition of 5μl glycogen. The mixture was placed in -20°C for one hour. After high speed centrifugation (13000rpm/10min/4°C), the DNA pellet was washed once with 70% ice cold ethanol, dried and resuspended into 20μl of TE buffer. An aliquot of the DNA was quantitated and qualified by electrophoresis

on an 10 % polyacrylamide gel with ethidiun bromide staining, and the rest was stored at -20 ^{0}C until needed.

5. 5. 1. 3 Ligation

The pCR-Script^{TM} SK(+) cloning kit from stratagene (Stratagene, USA) was used to clone the PCR products of interest. Steps such as the ligation, transformation and plating were followed as described in manufacturer's direction with minor modification.

For ligation, the ideal ratio of insert-to-vector DNA is 5:1 to 10:1. To a microcentrifuge tube, the following components were added in order: 1μl of pCR-Script^{TM} cloning vector (10 ng/μl); 1μl of pCR-Script^{TM} 10 x reaction buffer; 0.5μl of 10 mM ATP; 2-4μl of recovered PCR product; 1μl of *Srf* I restriction enzyme (5 μL / μl); 1μl of T4 DNA ligase and distilled H_2O to a final volume of 10 μl. The mixture was incubated at room temperature for 1 hour, and then it was incubated at 65 ^{0}C for 10 minutes. The sample was stored on ice until transformation into *Escherichia coli* supercompetent cells.

5. 5. 1. 4 Transformation

For transformation, the supercompetent cells (from The pCR-Script^{TM} SK(+) cloning kit) were thawed on ice. 40μl of cells was aliquoted into a pre-chilled 15 ml Falcon 2059 polypropylene tube. 0.7μl of the β-mercaptoethanol

provided with this kit was added to the 40 μl of bacteria to yield a final concentration of 25 mM. DNA of the ligation mixture 2μl was added to the 40μl of cells and swirled gently, then the reaction was placed on ice for 30 minutes. Heat shock was performed on the transformation mixture in a 42 ^{0}C water bath for 45 seconds, then it was placed on ice for 2 minutes. Preheated (42 ^{0}C) SOC medium 2 ml was added into the transformation mixture and it was incubated at 37 ^{0}C for 1 hour with shaking at 200 rpm.

5. 5. 1. 5 Plating

Aliquots of 50, 100, 150 and 190 μl of the transformation mixture were plated onto the LB-Ampicillin containing agar plates (50 μg/ml of Ampicillin) with X-gal (20μl of 10% (w/v) X-gal) and IPTG (20μl of 0.2 M IPTG). Using a sterile spreader, the mixture was spread evenly on the surface of the plates and the agar plates were incubated inversely overnight at 37 ^{0}C .

5. 5. 1. 6 Screening of recombinant plasmid

The cloned plasmid colonies were analyzed by restriction enzyme digestion, PCR and the DNA inserts were confirmed by DNA sequencing.

5. 5. 1. 6. 1 Cultures of Transformants

Colonies of the transformants appeared on the agar plates were picked with a flame-sterilized inoculating loop and streaked directly on the Ampicillin LB agar plate to check for the presence of Ampicillin-resistance marker. After overnight incubation at 37^0C, single-isolated colonies were inoculated 2 ml of fresh LB broth in a 15 ml Falcon 2057 tube and incubated at 37^0C with good aeration (shaking at 200 rpm) for overnight.

5. 5. 1. 6. 2 Minipreparation of plasmid DNA by standard protocol

After overnight culture, 1.5 ml overnight culture cells were pelleted by centrifugation (6,500 rpm/5 min/ RT) and then resuspensed in 100μl of Solution I (50 mM glucose, 25 mM Tris-base pH7.9 and 10 mM EDTA) then 200 ul of Solution II (0.2N NaOH, 1% SDS) was added and mixed by inverting the tube several times. Solution III (3 M potassium acetate, 2M glacial acetic acid) 150μl was added and mixed by inverting the tube and chilled on ice for 10 min. The supernatant was then transferred into a microfuge tube after centrifugation (13000rpm/10min/4°C). The supernatant was extracted by 500μl of phenol/chroloform/isoamyl alcohol (25:24:1)(v/v/v) for 1 minute by votexing. The aqueous (top) phase was transferred into a 1.5 ml eppendorf tube and of absolute ethanol was added to the aqueous phase and mixed by inversion for several times. The plasmid DNA was pelleted by centrifugation at 13000 rpm/4 min/RT. The supernatant was decanted and the pellet was washed with 70% ice

cold ethanol and lyophilized. Finally, the DNA was resuspended into 50μl of TE buffer and ready for further restriction analysis and PCR amplification.

5. 5. 1. 6. 3 Minipreparation of plasmid DNA by Magic Minipreps DNA purification system

The plasmid DNA was purified by Magic Minipreps DNA Purification system (Promega Corporation, Madison, WI, USA). 1.5 ml bacteria culture was transferred to a 1.5 ml eppendorf tube and pelleted by centrifuged at 6500 rpm for 5 minutes. The pellet was resuspended into 200μl of Cell Resuspension Solution and then mixed with 200μl of Cell Lysis Solution by inverting the tube several times until the solution clear. The lysate was neutralized by 200μl of Neutralization Solution and centrifuge at 13,000 rpm for 5 minutes. The cleared supernatant was decanted to a new microcentrifuge tube and 1ml of the Magic Minipreps DNA Purification Resin was added and mixed with the supernatant. The resin/DNA mix was pipetted into syringe barrel and passed through the Minicolumn by a vacuum . The Minicolumn was washed with Column Wash Solution and dried by applying a vacuum and centrifugation (13,000 rpm / 20 sec) to remove any residual Column Wash Solution. The plasmid DNA retained in the Minicolumn was eluted by 50μl TE buffer and the plasmid DNA was stored at -20°C for later analysis. The plasmid DNA prepared by this method was used for DNA sequencing.

5. 5. 1. 6. 4 Screening of recombinants by restriction enzyme digestion

After minipreparation of plasmid DNA, the plasmid DNA was digested by double restriction enzymes *Hind*III and *Sac*II which had the cutting sites on the vector in each side of the inserted DNA fragments. The restricted DNA was subjected to electrophoresis on 1% agarose gel followed by staining with ethidium bromide and visualized under UV light. An additional DNA fragment with the expected size indicated this clone contained the insert of the PCR product. The vector plasmid DNA restricted with *Hind*III and *Bam*HI and the PCR product were used as references. λ DNA/*Hind*III fragments and ϕX174 RF DNA/*Hae*III fragments were used as molecular weight markers.

5. 5. 1. 6. 5 Screening of recombinants by PCR

Alternatively, the plasmid DNA obtained from candidate clones through the Minipreparation were subjected to PCR. Using the corresponding sets of primers in the first round of PCR and with the same conditions, the plasmids DNA was amplified to determine if it contained the insert after agarose gel electrophoresis of PCR product. The presence of the right band indicated the clone containing the insert interested.

At least four positive clones from each cases were analyzed and the plasmid DNA was subjected to further analysis by DNA sequencing.

5. 5. 1. 7 Confirmation of cloned DNA by sequencing

ΔTaq cycle-sequencing kit (United States Biochemical) was used to sequence *p16* and *p15* exon 1 fragments inserted in the plasmid (pCR-Script™ SK(+) vector). The manufacture's directions were followed with minor modification. DNA amplification and sequencing reactions were carried out on all samples twice to eliminate errors due to possible contamination which might occur during the amplification reactions.

5. 5. 1. 7. 1 Primers used in sequencing

For sequencing cloned DNA, primers for the T7 and T3 RNA polymerase promoters located on the vector in the up-stream or down-stream of the inserts were used as sequencing primers. If necessary, primers (the PCR amplification primes) located in the insert were also used as sequencing primers (Table 3). DNA sequencing for the cloned inserts was performed by using the ΔTaq cycle-sequencing kit (United States Biochemical) .

5. 5. 1. 7. 2 End-labeling sequencing primer

For DNA sequencing, both the upstream or the downstream sequencing primers of the corresponding DNA fragment were labeled at their 5'-ends. The 10 pmol of primer was labeled with 2.5 μl [γ-^{32}P] ATP(5000Ci/μl, Amersham) by

10 units of T4 polynucleotide kinase in a total volume of 10 μl of kinase buffer, and incubated at 37 ^{0}C for 30 minutes. To stop the reaction, the tube was placed in a 70 ^{0}C water bath for 10 minutes and then it was placed on ice until ready for use.

5. 5. 1. 7. 3 Cycle sequencing reactions

Labeled primer and template were repeatedly cycled through alternating periods of denaturing, annealing, extension and termination.

Four 0.5 ml microcentrifuge tubes were labeled as "A", "C", "G" or "T" for the respective termination, and 4μl of the corresponding Termination Mix was aliquoted into the appropriately labeled tube. The tubes were placed at 4 ^{0}C until needed.

For each DNA template to be sequenced, the following master mix were prepared containing DNA template (50μg /μl) 2μl; labeled primer 1μl; reaction buffer 2μl (260 mM Tris-HC, pH9.5, 65 mM $MgCl_2$); diluted ΔTaq DNA Polymerase (1 unit / μl) 2μl; sterile distilled H_2O 10μl to a total volume 17μl. Then 3.5μl of the master mix was aliquoted into each of the labeled tubes containing the termination mix. The evaporation of reaction mixture was also prevented by addition of a drop of mineral oil to each tube. The tubes were placed into a pre-heated (95 ^{0}C) thermal cycler for 5 minutes and then followed the cycling program for 40 cycles: 95 ^{0}C for 30 seconds; 58 ^{0}C for 1 minute; 72 ^{0}C for 30 seconds, and final elongation for 5 minutes.

After cycling reaction, 4μl of stop solution was added below the oil overlay to stop the reaction. Finally, 2 μl of the each sample was loaded onto the sequencing gel immediately after the sample was denatured at 95^0C for 5 minutes. The sequencing gel was electrophoresed under standard conditions.

5. 5. 1. 7. 4 Electrophoresis

The sequencing system (Model S2, GILCO/BRL Life Technology) was used to separate the radioactive labeled DNA fragments by 6% denaturing polyacrylamide gel containing 23% urea. The sequencing gel was pre-run at constant 1700 volts for 30-60 minutes until 60^0 C was reached. The labeled DNA was denatured at 95^0 C for 5 minutes and chilled on ice before loading. 2 μl of each sequencing mixture was loaded into the adjacent well of the gel in the order of A, C, G and T. The electrophoresis was performed at constant 1700 volts until the faster bromophenol blue dye reached the bottom of the gel.

5. 5. 1. 7. 5 Autoradiography

After removing the gel from the electrophoresis apparatus, it was transferred carefully to a supporting sheet of filter paper (Whatman 3MM No.1). Then it was covered with high-quality plastic wrap, and dried by using a vacuum gel dryer at 80^0 C for 1 h. Autoradiogram of the sequencing gel was obtained

after an overnight exposure of the dried gel to Kodak X-OMAT AR film (Eastman Kodak Company) with double intensify screen.

5. 5. 2 DNA Extraction and purification

5. 5. 2. 1 DNA Extraction from bone marrow aspirate and peripheral blood

Genomic DNA was extracted from bone marrow aspirate and peripheral blood by the phenol/chloroform method. The frozen marrow aspirate or blood samples were first thawed on room temperature then transferred to 15 ml Falcon 2059 centrifuge tube. The cells were pelleted by centrifugation at 3200 rpm for 10 minutes at room temperature, and the cells were washed three times with 1 X PBS until clear solution is achieved. The cell pellet was resuspended in 2 ml initial resuspension solution (0.35 M NaCl, 1.5 mM sodium citrate pH7.0, 0.5% SDS, 1 mM DTT) and then proteinase K was added to a final concentration of 100 μg / ml and mixed gently by invasion of the tube. After incubation overnight at 55^0 C, the solution was cooled and the cellular DNA was extracted by mixing gently with 2 ml of ice-cold phenol /chloroform/ isoamyl alcohol (25 : 24 : 1) (v/v/v) reagent for 10 minutes. The mixture was then spun at 4000 rpm for 20 minutes at room temperature. The upper aqueous phase was collected. DNA was precipitated by mixing with one-tenth volume of 3 M sodium acetate pH5.2, and two volumes of ice-cold absolute ethanol and placed on -20°C for 1 hour. The DNA precipitate was pelleted at 4000 rpm for 20 minutes, washed once with

70% ethanol (-20^{0}C), and finally dissolved in 2 ml of TE pH7.5. Trace of RNA present in the DNA sample was digested with 100μg / ml DNase-free RNase A for 1 hour at 37^{0}C. The enzyme was inactivated by addition of the proteinase K at final concentration 100 μg/ml together with 0.5% SDS for 1 hour at 55^{0}C. The DNA was again extracted with 2 ml phenol/chloroform/isoamyl alcohol (25:24:1) (v/v/v) reagent, precipitated with two times volume of absolute ethanol, and dissolved in 500μl TE pH7.5. After checking and measuring the quality and quantity of DNA, the DNA samples were stored at -20^{0}C for later analysis. Special cares were taken to avoid cross-contamination among the samples.

5. 5. 2. 2 Isolation of plasmid DNA from transformant cultures

5. 5. 2. 2. 1 Cultures of Transformants

Colonies of the transformants appeared on the agar plates were picked with a flame-sterilized inoculating loop and streaked directly on the Ampicillin LB agar plate to check for the presence of Ampicillin-resistance marker. After overnight incubation at 37^{0}C, single-isolated colonies were inoculated 2 ml of fresh LB broth in a 15 ml tube and incubated at 37^{0}C with good aeration for 2 to 3 hr . Then 0.5 ml of the culture was inoculated into 200 ml of fresh LB broth containing 50 μg /ml Ampicillin in a 500 ml conical flask. The culture was incubated at 37^{0}C with shaking (200 rpm) for 6 hours. In order to improve the yield of plasmid DNA, chloramphenicol was added to 200μg/ml, and the culture

was again incubated overnight with shaking (200 rpm) at 37^0C.

5. 5. 2. 2. 2 Extraction and purification of plasmid DNA (large scale preparation)

5. 5. 2. 2. 2. 1 Wizard Maxipres DNA purification system

Wizard Maxipreps DNA purification system was used for rapid extraction and purification of plasmid DNA containing *p16* and *p15* exon 1 probes. Extraction and purification procedures were followed as described by the manufacturer. In brief, 200 ml of cultured cells was pelleted by centrifugation at 3,000 rpm for 15 minutes at room temperature. The cell pellet was resuspended in 15 ml of Cell Resuspension Solution by pipetting up and down until no clumps were visible. 15 ml of Cell Lysis Solution was added to the suspension and mixed gently, but thoroughly, by stirring or inverting until the solution became clear and viscous (about 10 minutes). And then 15 ml of Neutralization Solution was added and mixed immediately by gently inverting the tube several times. The final mixture was centrifuged at 4,000 rpm for 15 minutes at 22-25°C in a room temperature rotor. The cleared supernatant was transferred to two 50 ml Falcon tubes by filtering through a filter paper (Whatman #1) and mixed with 0.5 x volume of room temperature isopropanol. The mixture was again centrifuged at 13,000 rpm for 15 minutes at 22-25°C in a room temperature rotor. The supernatant was discarded and the DNA pellet was resuspended in 2 ml of TE

buffer. 10 ml of Wizard Maxipreps DNA purification Resin was added to the DNA suspension above and mixed by swirling. Transfer the Resin/DNA mix into the Maxicolumn by applying a vacuum to pull the Resin/DNA into the Maxicolumn. The Maxicolumn was washed twice with 12 ml Column Wash Solution and once with 5 ml of 80% ethanol, dried by centrifugation at 2,000 rpm for 5 minutes. The plasmid DNA was eluted by applying 1.5 ml of preheated (65-70°C) TE buffer to the Maxicolumn and centrifuging at 2000 rpm for 5 minutes. The purified plasmid DNA was stored at -20°C for later use.

5. 5. 2. 2. 2. 2 Standard protocol

Cells of the choromphenicol-amplified culture were harvested by centrifugation at 4000 rpm for 20 minutes. Ten ml of lysis buffer (2 mg/ml lysozyme, 25 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0 and 50 mM Glucose) was added to resuspend the cells, followed by the addition of 20 ml of alkali-SDS solution(1% SDS and 0.2 N NaOH). The solution was mixed and kept on ice for 5 minutes. Then 10 ml of 4M sodium acetate (pH 4.8) was added and incubated on ice for another 15 - 30 minutes. The mixture was mixed occasionally. After spinning at 12,000 rpm, at 4°C for 30 minutes, the supernatant was decanted into a clean 100 ml centrifuge tube. One volume of isopropanol and 1/10 volume of 3M sodium(pH 4.8) acetate were added and the mixture was left on -20°C for 1 hour. The sample was again centrifuged at 5000 rpm, for 10 minutes at 10°C. Then the supernatant was discarded, and the

pellet was resuspended in 5 ml of RNase buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0 and 10 μg/ml RNase). After incubation at 37^0 C for 15 minutes, DNA was precipitated by adding 0.1 volume of 5 M NaCl and 10 ml of ethanol. The DNA pellet was then collected by centrifugation at 10,000 rpm, 2 minutes, and resuspended with 10 ml of RNase buffer. The DNA solution was clarified by centrifugation at 5,000 rpm for 10 minutes. The soluble plasmid DNA was extracted with equal volume phenol/chloroform/ isoamyl alcohol solution, precipitated with ethanol, and dissolved in TE pH7.5. buffer. The quantity and quality of the plasmid DNA were determined spectrophotometrically and the DNA was stored at -20°C for later use.

5. 5. 2. 3 Checking of the quality and quantity of plasmid DNA

The quality of the extracted DNA was analyzed by gel electrophoresis through a 0.3% agarose gel poured on a 1% agarose support (the plasmid DNA was analyzed on 1% agarose gel).

The yield and purity of the DNA were determined by measuring the absorbance of the diluted DNA sample at 260 nm, 280nm and 330nm using a Phamacia spectrophotometer.

Purity Check: The ratio of (A260-A330)/ (A280-A330) =1.8 to 2.0 is the a acceptable parameter for DNA.

The ratio below this range indicates small amount of protein remaining in the sample. Ratio above this range represents the presence of RNA remaining in the samples.

DNA concentration (μg / ml): DNA conc. = (A260-A330) x DF x 50

where A260, A280 and A330 are absorbance at respectively 260 nm, 280 nm and 330 nm; and DF is the dilution factor. One unit absorbance at 260 nm equals 50 μg / ml DNA .

5. 5. 3 Detection of hypermethlation by Southern blot analysis

5. 5. 3. 1 Restriction enzyme digestion

For Southern-blot analysis, each 5μg DNA extracted from bone marrow aspirate and peripheral blood was first digested with 30 units of methylation-sensitive restriction enzyme *Sac*II, *Eag*I or *Sma*I separately at 37^0C overnight (30^0C for *Sma*I) according to the recommendations of the suppliers. The restricted DNA was ethanol precipitated . After centrifugation, washing and drying, the resulting pellet was dissolved in TE buffer and further digested with 30 units *EcoR*I or *Hind*III at 37^0C overnight. For reference, DNA from an unmethylated normal blood restricted with *EcoR*I or *Hind*III alone were used as positive control for the methylation, and the DNA restricted with *EcoR*I or *Hind*III plus one of the methylation sensitive enzymes (*Eag*I, *Sac*II and *Sma*I) was used as negative control for the detection of *p16* and *p15* methylation in Southern analysis. All twenty-four MM DNA were restricted with *EcoR*I or *Hind*III plus one

of the methylation sensitive enzymes (*Eag*I, *Sac*II and *Sma*I).

5. 5. 3. 2 Agarose gel electrophoresis

After addition of loading dye, the DNA samples were fractionated by gel electrophoresis for 8 hours at 50 V using 1% agarose gel in 1X TBE buffer. Molecular size marker consisted of *Hind*III-digested bacteriophage λ DNA fragments was used. The gel was stained with ethidium bromide (0.1μg / ml) for 15 minutes and then visualized and photographed under UV light.

5. 5. 3. 3 Southern transfer

The gel was denatured in denaturation solution (1.5M NaCl, 0.5M NaOH) for 45 minutes with gently shaking and neutralized by neutralization solution (3M NaCl, 0.5M Tris pH7.0) for another 45 minutes. The DNA was transferred to Hybon+ Nylon membranes (Amersham Corp. Hong Kong) by capillary action overnight.

5. 5. 3. 4 Membrane fixation

5. 5. 3. 4. 1 UV cross-linking

The membrane was allowed to air dry for an hour or dry at 80°C for 10 minutes and wrapped with Saran Wrap and then placed DNA-side down on a UV transilluminator for 2 minutes exposure.

5. 5. 3. 4. 2 Alkali fixation

The membrane was placed DNA side up on a pad of absorbent filter paper (Whatman No.1) 2-3 pieces thick soaked in 0.4M NaOH. After 20 minutes soaking, the membrane was rinsed by immersion in 5 x SSC or 5 x SSPE with gentle agitation for 1 minute. The membrane was sealed into a plastic hybridization bag (GILCO/BRL Life Technology) and ready for the hybridization step.

5. 5. 3. 5 Recovery and purification of *p16 and p15* exon 1 probe from plasmid

The PCR generated *p16* and *p15* exon 1 probes were released from the cloning vector pCR-Script™ SK(+) by digestion with the restriction endonuclease *Sac*II and *Hind*III under the conditions recommended by the manufacturer.

Typically, in a reaction volume of 200 μl , 200μg of plasmid DNA was mixed with 200 units of restriction enzyme in 1X reaction buffer. The reaction mixture was incubated at 37°C overnight

The restricted DNA was mixed with loading buffer consisting of glycerol, xylene cyanol FF and bromophenol blue loaded into the wells of a 1% agarose gel containing 0.1 μg / ml ethidium bromide. The electrophoresis was performed at 50 volts for 3 hours in 1X TBE in a horizontal gel electrophoresis system (Gilco/BRL Life Technology). The *Hind* III digested λ-DNA marker was also run on the same agarose gel parallel with the DNA samples. The electrophoretic pattern was visualized under UV illumination (UVP Transilluminator Benchtop, UVP Inc. Ultra-violet Products, Inc.), and *EcoR*I by a Polariod DS34 direct screen instant camera using Polaroid 667 film (ASA 3000). The DNA band of interest was cut out with a sharp scalpel, minced and then transferred to 1.5 ml eppendrof tube with 0.5 ml TE buffer. The DNA probes were eluted out of the gel onto TE buffer by incubation in 37°C water bath overnight. After spinning at high speed for 1 minute in a microcentrifuge to pellet the pieces of agarose gel, the supernatant was collected in a clean microcentrifuge tube. The eluted DNA probes were concentrated by precipitation with one tenth volume of 3 M sodium acetate (pH 5.2) and 2X volume of absolute ethanol with addition of 5 μl of glycogen. The mixture was placed at -20°C for one hour. The identity and semi-quantity of an aliquot of the DNA probes were confirmed and estimated by gel electrophoresis through an 10 % polyacrylamide mini-gel (Gilco/BRL Life Technology). The DNA fragments were then stored at -20^{0}C.

5. 5. 3. 6 Probe labeling

The *p16* and *p15* exon 1 fragments isolated from plasmid were labeled by rediprime DNA labeling system (Amersham Life Science). The DNA fragments to be labeled were diluted to 25 ng in 45μl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and denatured by heating to 95-100°C for 5 minutes in a boiling water bath. The denatured DNA was added to the labeling mix and reconstituted the mix by gently flicking the tube until the blue color was even distributed. Then 5 μl of Redivue [α- ^{32}P] dCTP (3000Ci/mmol; Amersham Life Science) was mixed with reaction mixture and incubated at 37°C for 10 minutes and followed by adding 5μl of 0.2M EDTA to stop the reaction. The labeled probes were denatured by heating to 95-100°C for 5 minutes, then chilling on ice for later hybridization step.

5. 5. 3. 7 Purification of radioactive labeled DNA probes by spun-column

In order to minimize the degree of non-specific binding during the hybridization, the un-incorporated [α-^{32}P] dCTP was removed together with the unlabelled deoxynucleotides in the rediprime enzyme / buffer mixture.

For the preparation of Spun-column, a 1.0 ml syringe (Japan Medical Supply Co., LTD) was plugged with a small piece of siliconized glass wool and then placed in an inserted. Equilibrated Sephadex G50 (Pharmacia AB

Molecular Biology) in STE was allowed to fill the syringe and then it was packed down by centrifugation at 3500 rpm for 4 minutes. The syringe was re-filled with Sephadex G50 and centrifugation were repeated until the column was packed to a volume of 1 ml and then the column was washed with 0.1 ml of STE buffer 3 times. After washing, 100μl of radioactive labeled DNA was applied onto the column. The effluent containing purified radioactive labeled DNA probes (100μl) was collected in a fresh decapped 1.5 ml eppendorf tube by centrifugation at 3500 rpm for 4 minutes as above.

5. 5. 3. 8 Southern hybridization

Southern hybridization was performed in Rapid-hyb buffer (Amersham Life Science). The membrane was prehybridized with pre-warm rapid hybridization buffer at 65°C with shaking for at least 30 minutes. The labeled and purified DNA probe plus herring sperm DNA (100μg/ml of buffer) was denatured at 95-100°C for 10 minutes. A sufficient amount of labeled probe about 5 x 10^6 cpm / ml buffer of labeled probe was added to the hybridization buffer and the hybridization was carried out at 65°C with shaking for 2.5 hours.

5. 5. 3. 9 Post hybridization wash

After hybridization, the membrane was washed twice at room temperature for 10 minutes in 2 x SSC / 0.1% SDS, twice at 65°C for 10 minutes

in 1 x SSC / 0.1% SDS followed by three times at 65°C for 15 minutes washes in 0.1 x SSC / 0.1% SDS with shaking.

5. 5. 3. 10 Autoradiography

The membranes were wrapped in SaranWrap and exposed to Kodak X-OMAT AR film (Eastman Kodar Company, Rochester, NY) in light-tight cassettes with two intensifying screens at -70^{0}C for 1-5 days.

5. 5. 4 Polymerase Chain Reaction-Single Strand Conformational polymorphism Analysis (PCR-SSCP)

In PCR-SSCP analysis, the target sequence in genomic DNA was simultaneously amplified and labeled by using radioactive labeled primers. The amplified product was then denatured to a single-stranded form and subjected to non-denaturing polyacrylamide gel electrophoresis. Bands of the single-stranded DNA at different positions in the autoradiogram indicate the presence of mutations.

5. 5. 4. 1 5' - end radioactive labeling of primer

In the presence study, 5'-end labeling kit (Amersham) was employed to radioactively labeled primers with [γ-^{32}P] ATP (3000Ci/mmol) (Amersham)

A pair of upstream and downstream primers (5 pmol each) flanking each

exon of *p16* and *p15* was phophorylated with 5μl of [γ-^{32}P] ATP by 10 units of T4 polynucleotide kinase in a total volume of 10μl phosphorylation reaction buffer incubated at 37°C for 30 minutes. These amount of radioactive labeled primers was enough for amplifications of target sequences in 40 DNA samples.

5. 5. 4. 2 Amplification of target sequence by PCR

The Taq DNA Polymerase (GILCO/BRL Life Technology) was used in this study for *in vitro* amplification of target sequence.

The radioactive labeled primers solution was diluted with 105 μl of autoclaved double distilled water. In the PCR reaction mixture for 40 DNA samples, 20 μl of 10X PCR buffer and 5μl of 2.5 mM deoxyribonucleotide triphosphates (dNTP) mixture was added. After addition of 5 units of Taq DNA polymerase, 4μl of this PCR reaction mixture was aliquoted to forty 0.5 ml thin wall PCR tubes containing 1μl (about 100 ng) of genomic DNA sample as DNA template in each tube and then a drop of mineral oil was added to prevent the evaporation. The PCR was carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus) under the following conditions: the template DNA was first denatured by incubation for 5 minutes at 95°C followed by 40 cycles of PCR (1 min incubation at 60°C for annealing, 30 seconds at 72°C for polymerization and 30 seconds at 94°C for denaturation).

5. 5. 4. 3 Non-denaturing Polyacrylamid Gel Electrophoresis

The non-denaturing conditions, single-stranded DNA has a folded structure that is determined by intramolecular interactions in the nuleotide sequence. Different size and shape as well as its surface charge density of this particle caused different mobility in the non-denaturing polyacrylamide gel electrophoresis (Model S2, BRL).

Before electrophoresis, the 5μl of PCR product was mixed with 45μl of loading buffer and denatured by heating at 95°C for 10 minutes and chilling on ice. 2μl of the denatured sample was loaded on a 40 cm polyacrylamide (BRL)(49:1, acrylamide: N,N'-methylene bisacrylamide)/0.5X TBE gel with the combinations of 8% or 6% polyacrylamide gel with or without 5% glycerol added. The gel was run at two different conditions: 4°C/30 watts/4 hrs or 6 hrs in 0.5XTBE buffer and RT/1.5 watts/18hrs in 0.5XTBE buffer.

Finally, the gel was transferred onto Whatman 3MM No.1 paper and dried up using gel dryer (Model 583, GEL DRYER, BIO-RAD). Autoradiography was performed using an intensifying screen and an exposure of 12-48 hours at room temperature.

5. 5. 4. 4 Direct DNA sequencing of PCR product for mutation

Abnormal band with different mobility shift compared with the normal controls (normal blood and placental DNA) were excised from the dried gel, placed in 50μl of autoclaved double distilled water, and incubated at 65°C for

6 hours. Supposingly, only PCR products from the mutated allele can be obtained. After centrifugation at 16,000 rpm for 10 minutes (Micro Centaur MSE), 10μl of supernatant was collected for re-amplification of this eluted DNA with the corresponding sets of primers using the same conditions as described above in a total volume of 100 μl. Following 6 % polyacrylamide (29:1, acrylamide: N,N'- methylenebisacrylamide)/1XTBE gel electrophoresis (Model V16-2, Gilco/BRL), the slice of the gel with re-amplified PCR product was cut, minced and soaked in 500 μl of TE buffer pH7.5 overnight at 37°C to release the DNA from the gel. In order to prevent contamination of the gel in the DNA sequencing reaction, the supernatant was collected for DNA extraction by phenol/chloroform method after spinning down all the minced gel. The re-amplified PCR product was then precipitated by 2 volume of absolute ethanol at -20°C. The DNA pellet was dissolved in 20μl of autoclaved double distilled water. This purified DNA was ready to be used for direct DNA sequencing using ΔTaq™ Cycle-Sequencing Kit (United State Biochemical).

For direct sequencing, either upstream or down stream sequencing primer of the corresponding exon of the *p16* and *p15* was radioactive labeled by 5'-end labeling kit as above. The primers used for sequencing were the same primers for PCR-SSCP analysis and listed in Table 3. The labeled primer was diluted one fold by autoclaved double distilled water before addition to sequencing reaction mixture.

In the cocktail of DNA sequencing reaction mixture, 1μl of radioactive labeled primer was mixed with 2μl of re-amplified PCR product as template DNA,

2μl of reaction buffer and 2μl of ΔTaq version 2.0 polymerase in total volume of 17μl. Then 3.5μl of this cocktail was aliquoted to 4 termination tubes labeled with "A", "G", "C", and "T". Each tube contained 4μl of one of the Termination Mix. For example, the "G" labeled termination tube was filled with 4μl of ddGTP Termination Mix. The evaporation of reaction mixture was also prevented by addition of a drop of mineral oil to each tube. The termination reactions carried out in a DNA Thermal Cycler using the sample condition for PCR - SSCP analysis.

The addition of 4μl of stop solution was followed to stop the termination reaction. Finally, the sample was boiled for 10 minutes and 2μl of it was immediately loaded on the 8% sequencing gel which was pre-run at 1700 volts for 30-60 minutes until 60°C was reached. After electrophoresis at 1700 volts for 2 hours, the sequencing gel was blotted on to Whatman 3 MM No.1 paper, dried in gel dryer and exposed to Kodak AR X-ray film (Eastman) at room temperature for 12-48 hours with an intensifying screen.

5. 5. 5 Prevention of overall contamination in PCR

PCR is capable of amplifying a single copy of target DNA . Therefore, a major concern about this methodology is its susceptibility to contamination from other genomic DNA during preparation of samples or PCR reactions. Contamination may occur by three ways: sample to sample carry over; carry over of amplified DNA from previous PCR; and contamination from sample or product

to PCR-reagents. To minimize potential errors due to carry over several procedures can be adopted. These include the physical separation of pre- and post-PCR procedures and performing the procedure in a laminar flow hood to reduce contaminating DNA by aerosols and to irradiation.

Reagents were aliquoted and stored in an area free from amplified DNA. By labeling each lot and recording the usage of reagents, it was possible to track down contamination. Positive displace pipettes or filtered tips were able to reduce contamination from pipettes to samples. Precautions in the handling of all stages from sample collection, preparation, and extraction helped prevent cross-contamination. These include the frequent changing of gloves, minimizing the handling of samples, the addition of non -sample reagents prior to the addition of DNA sample, frequent cleaning of equipment and work area to remove nucleic acid, and also care to reduce the production of aerosols. Separate rooms for pre- and post -amplification procedures.

Aerosols are particularly important sources of contamination as particles can be as large as 20 μm in diameter ($4x10^{-4}$μl) and may carry $2.4x10^{4}$ copies of an amplified sequence. Appropriate controls were included in each batch of PCR and if any contamination was detected, results were repeated after de-contamination.

Human placenta tissues as a negative control sample was subjected to the same extraction and purification procedures as the multiple myeloma samples. For every sample preparation and extraction series, at least as many negative control samples were included as primer pairs used.

For the detection of infectious agents, it was always necessary to check the patients sample for the presence of amplifiable DNA. This was done with primers that amplify a human β-globin sequence to check extracted DNA integrity. If the a sample can not be amplified by β-globin primers, the sample will be excluded for experiment.

5. 5. 6 Sensitivity, specificity controls.

To assess the specificity of each primer set, the amplified PCR products obtained from patient's DNA and normal blood DNA were analyzed by DNA sequencing.

In addition, a negative control (buffer without sample) was included in every run. Results were considered valid only if they were consistent in at least two tests on samples derived from two independent extraction, and twelve normal bloods and one normal bone marrow aspirate were used as negative controls.

Primers for human β-globin gene was also used as an internal control to test the quality of the purified cellular DNA for PCR. PCR of the β-globin gene, a product a of 268 bp, was used to assess the quality and the internal control of the PCR reaction. Amplification parameters were as follows : Aliquots (1-10μl) of prepared samples are amplified separately with primer pairs PC04/GH201. Each 20μl reaction should contain 50 ng of sample DNA; 1μM of each primer; 200 μM of each dNTP (dATP, dGTP, dCTP, dTTP); 1 units Taq

DNA polymerase (Gilco/BRL Life Technology) 1.5 mM $MgCl_2$, and in 1X PCR buffer. Cycling parameters were 1 minutes at 95^0C, 1 minutes at 56^0C and 2 minutes at 72^0C for 35 cycles, followed by an additional 5 minutes extension at 72^0C on DNA thermal cycler (Perkin Elmer/Cetus).

Aliquots of PCR amplified DNA were resolved on 10% polyacrylamide gel, stained with ethidium bromide, and photographed under UV light. The results gave a indication of the quality of DNA.

Chapter 6

Results

6. 1 Patient characteristics

6. 1. 1 General patient characteristics

A total of twenty-four MM patients were studied. All except one (No. 7) were Chinese. Table 4 shows the clinical and laboratory profile of the 24 MM patients. Male-to-female ratio was 1.4:1 with the median age at 58.6 and range from 25 to 81 years old. It is noteworthy that one patient (No.20) was diagnosed at 25 years of age, which is extremely unusual for MM. The median follow-up duration was 24 months from the first consultation at PWH to the time of data analysis. Eight died and fifteen patients were alive at the time of writing. One patient (No.17) was lost to follow-up.

Our MM patients represent a group of patients with relatively advanced stage of the disease. Eighteen of them were at stage III disease and five at stage II and only one at stage I. Seventeen cases were analyzed on pre-treated samples.

6.1.2 Clinical and laboratory features

The diagnosis of MM was verified in each case with reference to the criteria from Durie and Salmon (2 and 4). Paraproteins fulfilling the major criteria by Durie were present in nineteen patients, sixteen of which in the serum and three in the urine. All except one (No. 5) patients had more than 30% of

three in the urine. All except one (No. 5) patients had more than 30% of myeloma cells in the bone marrow. Nineteen patients showed osteolytic bone lesions (Table 5A). Twenty-two patients had immunoparesis by protein electrophoresis. Three patients developed plasmacytomas as part of the myelomatosis process, one from the IgG isotype and two from the light chain disease (Table 4). Nine patients had renal failures although none showed hypercalcamia (Table 5A). Twenty-one patients had low albumins. Myeloma cells were identified through morphological assessment. They accounted for 25-100% of total nucleated cells present in the bone marrow. The complete blood counts showed a mean hemoglobin of 9.6 g/dL (range, 6.9-11.9), white blood count $4.4x10^9$/L (range, 2.9-7.1) and platelet count $161x10^9$/L (range, 52-432) (Table 5A).

The immunoglobulin isotypic profile showed that thirteen patients were of IgG, seven IgA, one IgD and three light chain disease (Table 5B). Table 5C shows the histological subtypes of our MM patients. Seven cases were classified as mature (Fig. 1A-B), nine as intermediate (Fig. 1C-D), seven as blastic (Fig. 1E-F) MM which also included one patient with anaplastic features (Fig. 1G-H). One patient showed atypical monocytoid myeloma cells.

Table 4 Clinical and laboratory profile of the 24 myeloma patients

Patient	Age/Sex	Dx	Ig	Pcytoma	Histology	Stage	R (f)	Alb	% BMPC	*P16*	*P15*	Tx
1	52 M	BILP	Gλ	–	Mature	III	N	LOW	90	M	M	MP
2 #	63 M	BILp	Dλ	–	Blastic	III	I	LOW	100	M	M	VAD→MP
3 #	42 F	BILP	Gλ	+	Intermediate	III	N	LOW	80	M	M	CEVAD *
4	48 M	BILP	Gλ	–	Mature	III	N	LOW	80	N	N	MP
5	38 M	bILPu	K	+	Monocytoid	I	N	LOW	25	M	M	RT→CEVAD
6	47 M	BILp	Aλ	–	Blastic **	III	N	LOW	50	M	M	CEVAD *
7	59 M	BILP	Gλ	–	Mature	III	N	LOW	40	N	N	MP
8 #	80 F	BILp	GK	–	Mature	III	I	LOW	90	M	M	RT + D
9	41 F	BIP	GK	–	Intermediate	II	N	LOW	35	N	N	CEVAD *
10	68 M	BILp	Aλ	–	Blastic	III	N	N	52	M	N	MP
11 #	61 M	BILPu	K	+	Blastic	II	I	N	90	M	M	MP + RT *
12	66 F	BIP	AK	–	Mature	III	I	LOW	32	M	M	M ± P
13	63 F	BILPs	AL	–	Mature	III	N	LOW	35	N	M	MP+RT*
14	75 F	BILPs	GK	–	Intermediate	III	N	LOW	36	M	M	MP+RT
15	49 M	Bps	AL	–	Blastic	II	N	LOW	35	M	N	MP
16 #	51 F	BILPs	GL	–	Blastic	III	N	LOW	84	M	M	MP
17	66 M	BLPs	GL	–	Intermediate	III	I	LOW	32	N	N	Defaulted
18	64 F	BIPu	L	–	Mature	III	I	N	63	N	N	Dex
19 #	81 M	BILPs	AK	–	Intermediate	III	I	LOW	80	M	M	MP
20	25 M	BILPs	GK	–	Intermediate	III	I	LOW	100	M	M	CEVAD
21	80 M	BILPs	GK	–	Blastic	III	N	LOW	90	M	N	MP
22 #	69 F	BIPs	AK	–	Intermediate	II	I	LOW	50	N	N	Dex
23 #	56 F	BILPs	GK	–	Intermediate	II	N	LOW	58	M	M	CEVAD*
24	63 M	BILPs	GK	+	Intermediate	III	N	LOW	50	N	N	MP*

: Dead ** : Anaplastic morphology * : Post-treated

Table 4 Clinical and laboratory profile of the 24 myeloma patients (continue)

Dx : Diagnosis
- B = myeloma cell infiltration > 30% of total nucleated cells (TNC) in bone marrow
- b = myeloma cell infiltration > 10%, < 30% of TNC in bone marrow
- I = immunoparesis, normal IgM<50mg%, IgA<80mg%, IgG<600mg%
- L = osteolytic lesions
- P = major paraprotein level (fulfill major criteria of Salman & Durie)
- p = minor paraprotein level (fulfill minor criteria of Salman & Durie)

Ig : Immunoglobulin isotype

Pcytoma : Plasmacytoma

R (f) : renal function, N = normal, I = impaired

Alb : Albumin, N = normal

%BMPC : % Bone marrow plasma cells

P16, P15 : M = methylation, N = normal

Tx : Treatment

M = Melphalan, P = Prednisolone, V = Vincristine, A = Adriamycin, D = Dexamethasone, C = Cyclophosphamide, E = Etoposide, RT = Radiotherapy

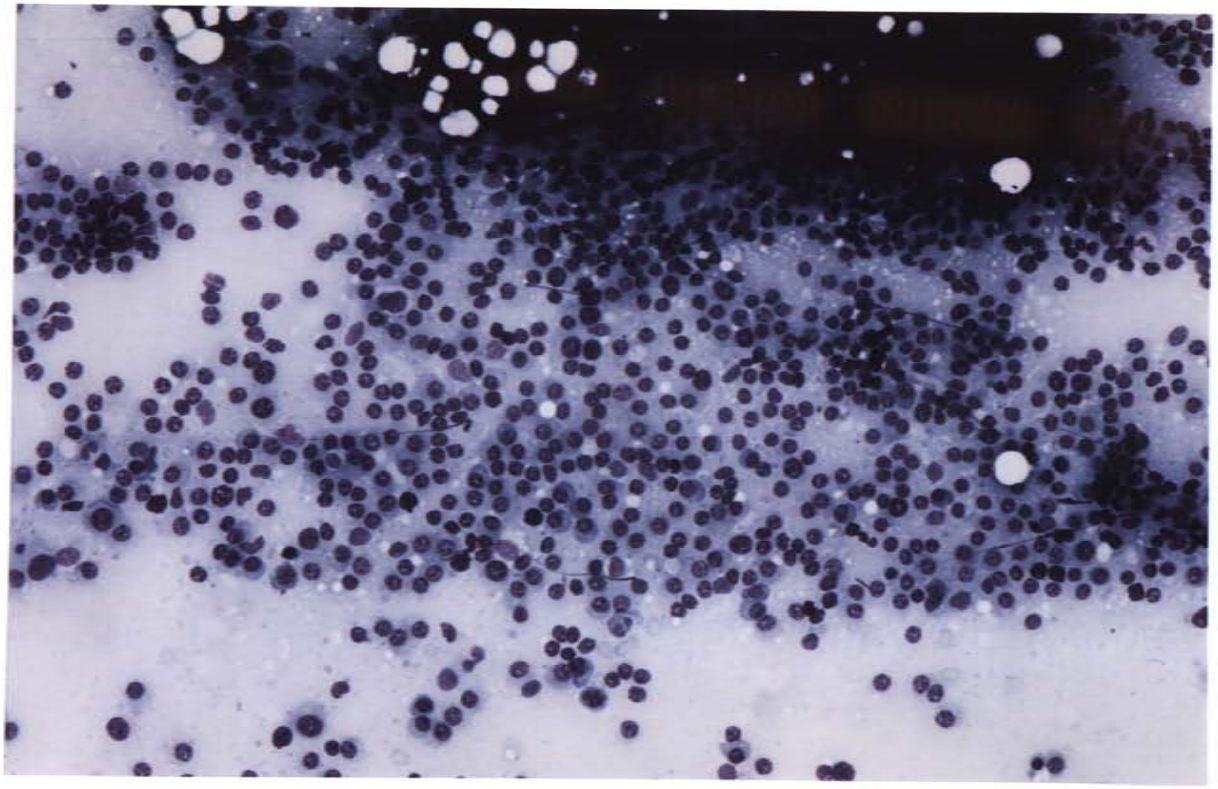


Fig. 1 (A) MGG stain of BM aspiration smear from a case of mature MM. The majority of the tumor cells are monomorphic, demonstrating typical cellular characteristics of plasma cells, namely 'clock-face' chromatin pattern, eccentric nucleus, distinct golgi zone and bluish cytoplasm. (200X)

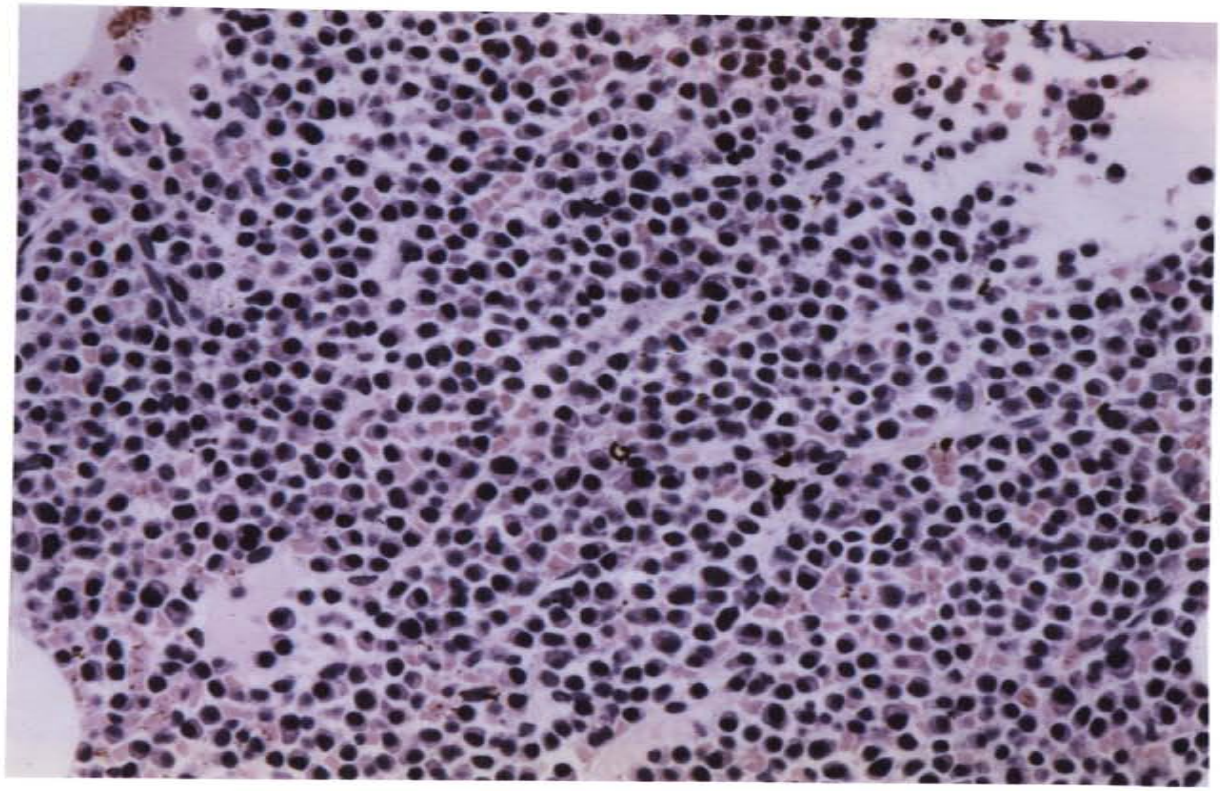


Fig. 1 (B) H & E section of trephine biopsy from a case of mature MM. The tumor cells are monomorphic in appearance with typical cellular features of a plasma cell. (400X)

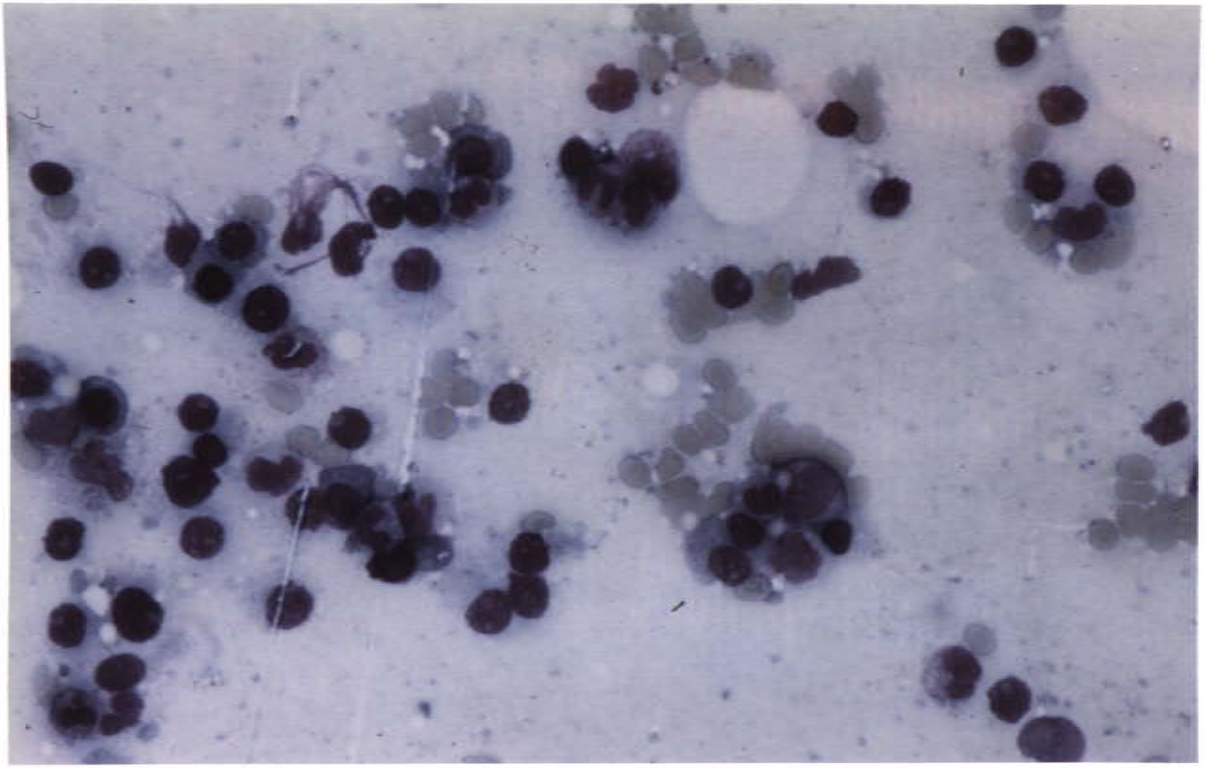


Fig. 1 (C) MGG stain of BM aspiration smear from a case of intermediate MM. A mixture of typical plasma cells and immature (blastic) myeloma cells is present. The immature myeloma cells exhibit a central prominent nucleolus, uncondensed chromatin and nuclear-cytoplasmic maturation asynchrony. (400X)

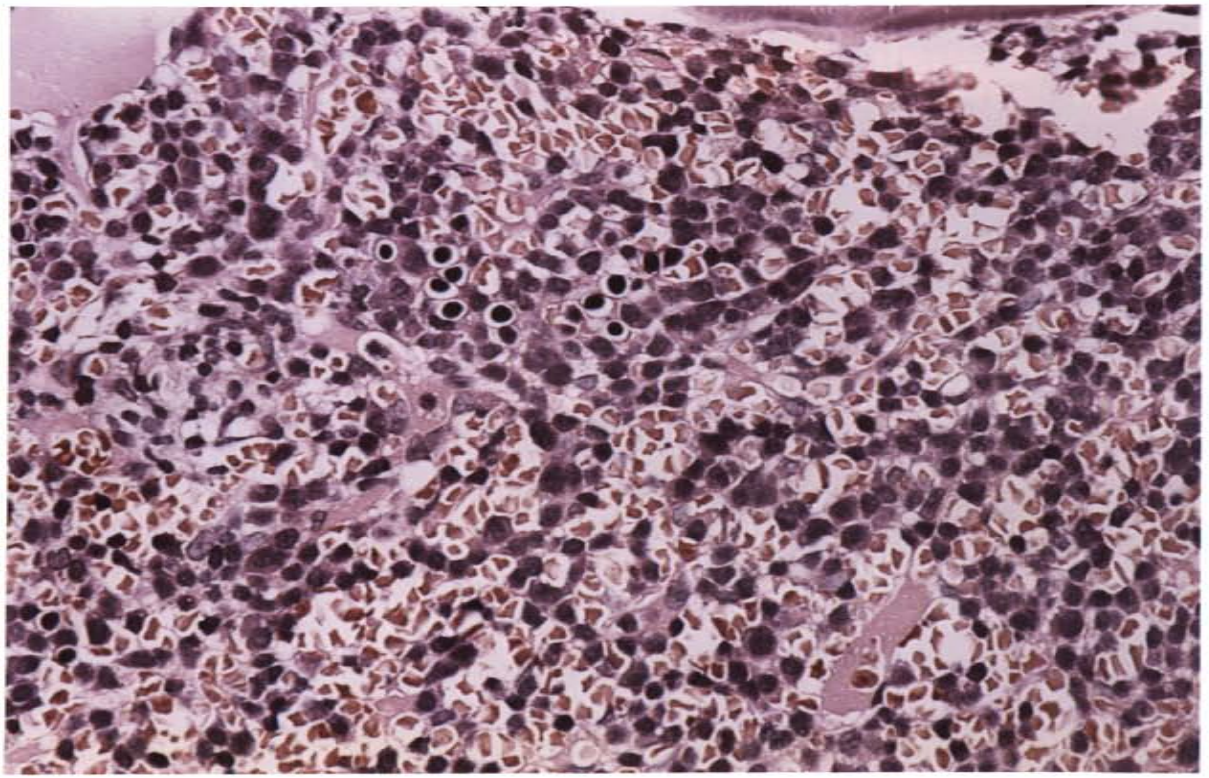


Fig. 1 (D) H & E section of trephine biopsy from a case of intermediate MM. A mixture of mature and immature myeloma cells are discerned. A few erythroblasts marked by perinuclear halos are present. (400X)

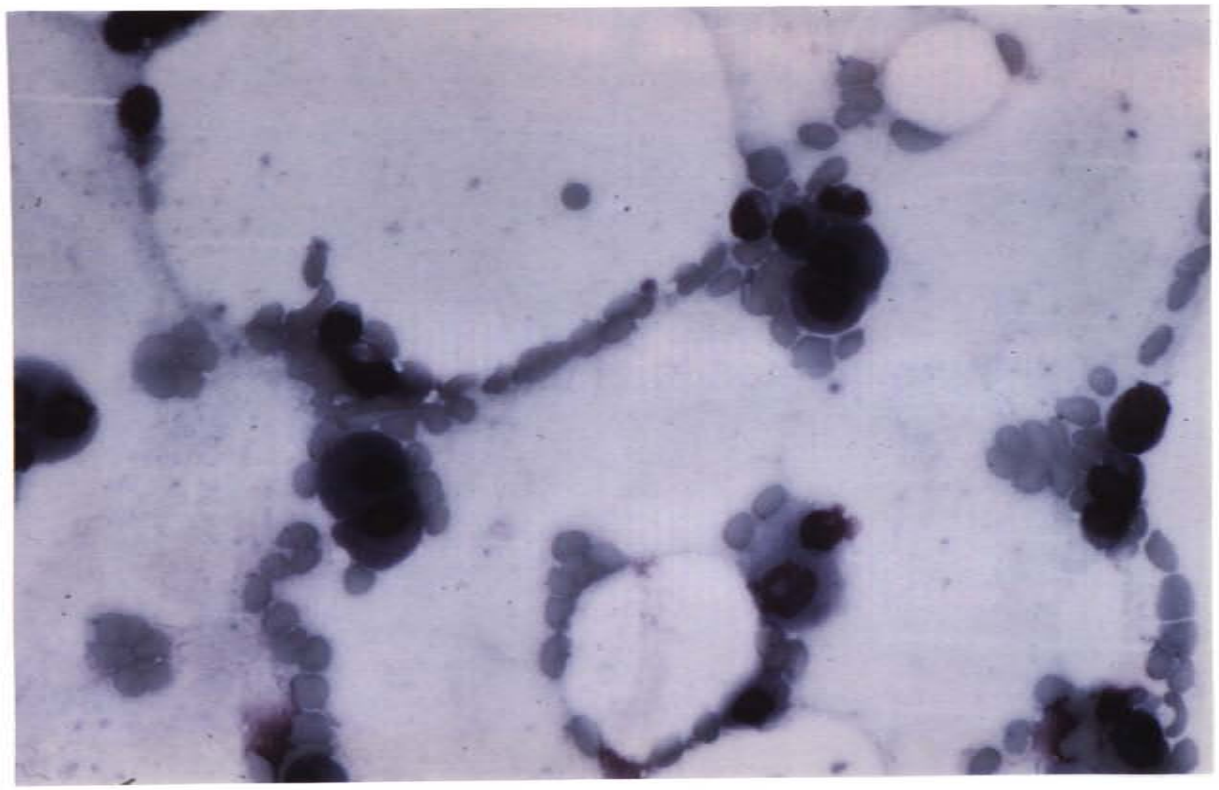


Fig. 1 (E) MGG stain of BM aspiration smear from a case of blastic MM. The scant tumor cells present are all characterized by single central prominent nucleolus, fine chromatin, abundant bluish cytoplasm with nuclear-cytoplasmic maturation asynchrony. (400X)

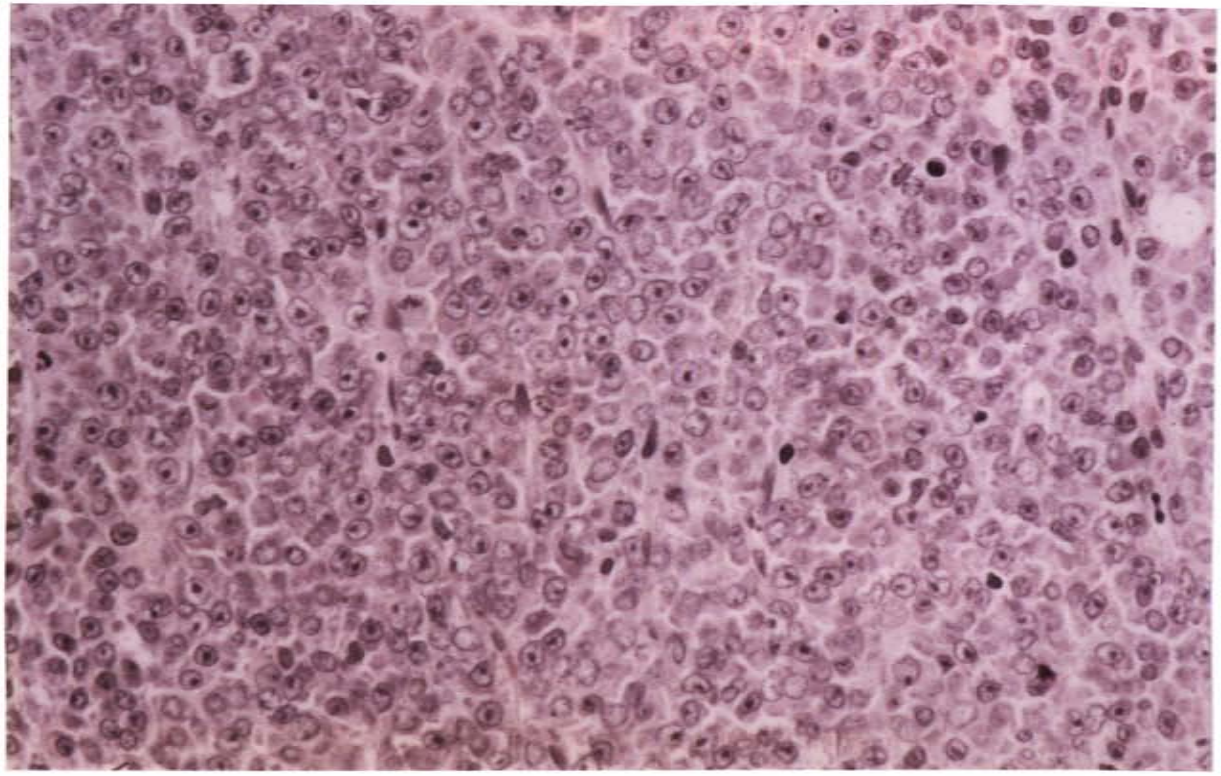


Fig. 1 (F) H & E section of trephine biopsy from a case of blastic MM. The tumor cells exhibit single and central eosinophilic nucleolus, thin nuclear border and fine chromatin. Occasional tumor cells show eccentric nuclei. Mitoses are also observed. (400X)

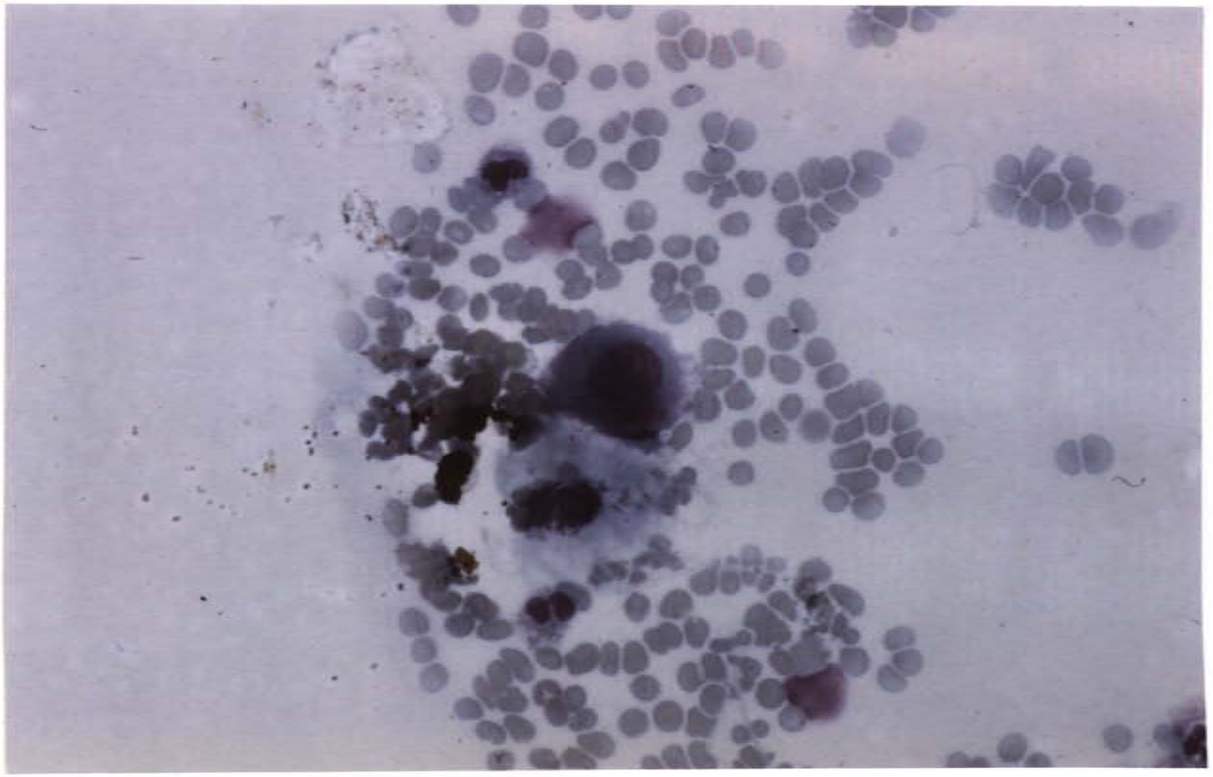


Fig. 1 (G) MGG stain BM aspiration smear from a case of anaplastic IgD MM. This is basically an aparticulate smear with very few anaplastic myeloma cells. The tumor cell shown is gigantic, binucleated, nucleolated with abundant bluish cytoplasm. (400X)

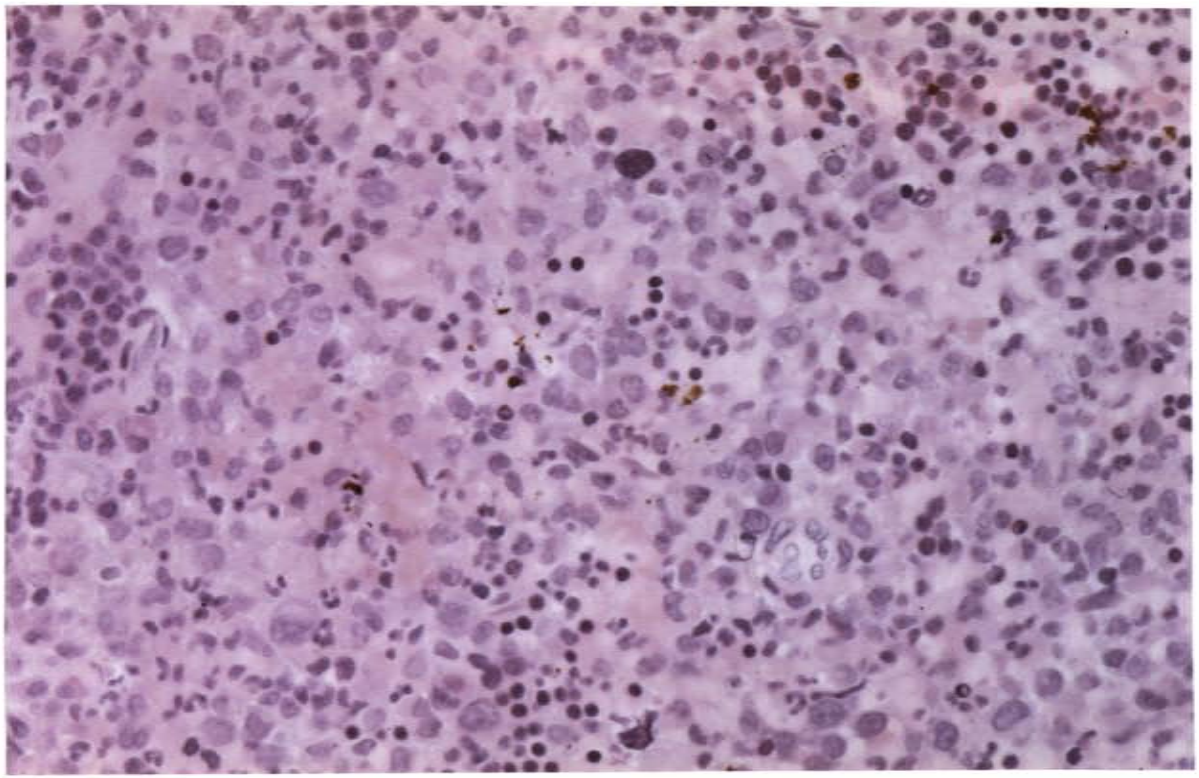


Fig. 1 (H) H & E section of trephine biopsy from the anaplastic IgD MM. The tumor cells are pleomorphic with irregular nuclear configuration. The residual hematopoietic cells are diffusely scattered among tumor cells. (400X)

Table 5A Clinical and laboratory features

Lytic lesions	79% (19/24)
Immunoparesis	92% (22/24)
Plasmacytomas	13% (3/24)
Renal failure	38% (9/24)
Hypercalcaemia	0% (0/24)
Hypoalbuminaemia	88% (21/24)
% BMPC	25-100%
Hb (g/dL)	6.9 – 11.9 (9.0 ± 1.41)
WBC (x 10^9/L)	2.9 – 9.6 (5.4 ± 1.85)
PLT (x10^9/L)	52 – 432 (208 ± 113)
Paraprotein	79% (67% S and 12% U)

BMPC: Bone marrow plasma cell
Hb : Hemoglobin
WBC : White blood cell
PLT : Platelet
S : Serum
U : Urine

Table 5B Profile of immunoglobulin isotypes

	Our Patients (N=24)	Others*
IgG	54%	56%
IgA	29%	26%
IgD	4%	1.5%
LC	13%	15%
IgM	0%	0.2%
NS	0%	1.1%

* Data from Western literature (Galton DAG: Myelomatosis. Postgraduate Hematology, Butterworth Heinemann, p. 474, 1989)

Table 5C Histological subtypes

Histological Subtypes	MM patients (N=23)
Mature	30%
Intermediate	39%
Blastic	46%

* One case excluded as it is typed as classified monocytoid myeloma

6. 2 Southern blot analysis of *p16/p15* and *Rb*

6. 2. 1 Absence of deletions or hypermethylation in normal controls

The presence of 5'CpG islands located around the transcription start sites of both *p16* and *p15* have been described previously (Herman et al, 1996; Gonzalez-Zulueta et al, 1995). Restriction of genomic DNA from bone marrow aspirates of our twenty four MM patients with the flanking enzyme *EcoR*I/*Hind*III, plus a methylation-sensitive enzyme (*Sac*II, *Eag*I or *Sma*I), on Southern hybridization with the exon 1 probes of *p16* and *p15* provided information on deletion and the methylation status of the 5'CpG islands of these genes (Merlo et al, 1995; Herman et al, 1996). As is typical of 5'CpG island in normal cell, twelve normal peripheral blood and one normal bone marrow aspirate samples tested showed no deletions or hypermethylation of the *p16* (Fig. 2A) and *p15* genes (data for *p15* not shown).

6.2.2. Absence of homozygous deletions or mutations in *p16/p15* and *Rb* among all MM patients

By Southern blot analysis, none of our MM patients had homozygous deletions of either *p16* or *p15* (Fig. 2B-D). The status of *Rb* was examined by two *Rb* cDNA probes (3.8 kb and 1.9 kb). No homozygous deletions or gene rearrangements were observed in our twenty-four MM patients (Table 6).

The exons 1, 2 & 3 for *p16* and exons 1 & 2 for *p15* were examined using PCR-SSCP and DNA sequence analysis. No mutations were identified in the coding

sequences of these genes (Table 6). All findings above were consistent with that in the literature.

Table 6 *p16, p15* and *Rb* gene alteration in multiple myeloma

Molecular analysis	Homozygous deletion		Methylation of *p16* gene			Methylation of *p15* gene	*p16* gene mutation (exon 1 - 3)	*p15* gene mutation (exon 1 - 2)	*Rb* gene	
	p16 gene	*p15* gene	*Sac*II	*Eag* I	*Sma*I	*Eag* I			rearragement	deletion
Patient No.										
1	-	-	+	+	-	+	-	-	-	-
2	-	-	+	+	-	+	-	-	-	-
3	-	-	+	+	-	+	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	+	-	-	+	-	-	-	-
6	-	-	+	-	-	+	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	+	+	-	+	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	+	+	-	-	-	-	-	-
11	-	-	+	+	-	+	-	-	-	-
12	-	-	+	+	-	+	-	-	-	-
13	-	-	-	-	-	+	-	-	-	-
14	-	-	+	+	-	+	-	-	-	-
15	-	-	+	+	-	-	-	-	-	-
16	-	-	+	+	-	+	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	+	+	-	+	-	-	-	-
20	-	-	+	+	-	+	-	-	-	-
21	-	-	+	+	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	-	-	+	+	-	+	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-

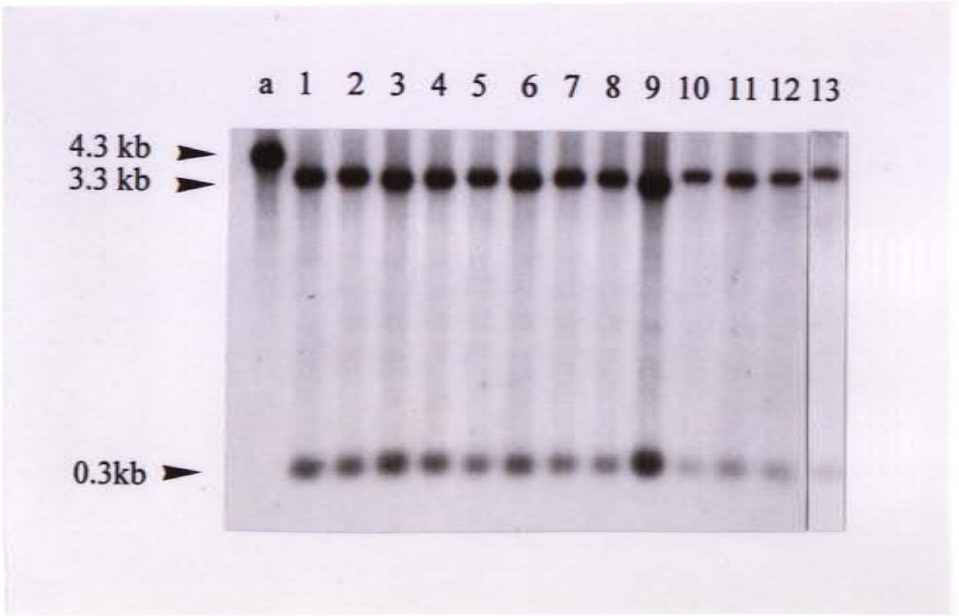


Fig. 2 (A) Southern blot analysis with the *p16* exon 1 probe for normal DNA samples (blood and bone marrow aspirate). Lane a is normal blood DNA restricted with *EcoR*I alone (the 4.3 kb band simulates the fragment size seen as if the *Sac*II sites are methylated). Lanes 1-12 (normal blood) and lane 13 (marrow) are DNA all restricted with *EcoR*I plus *Sac*II. All lanes show no methylation band but two smaller fragments of 3.3 and 0.3 kb, which indicates absence of methylation.

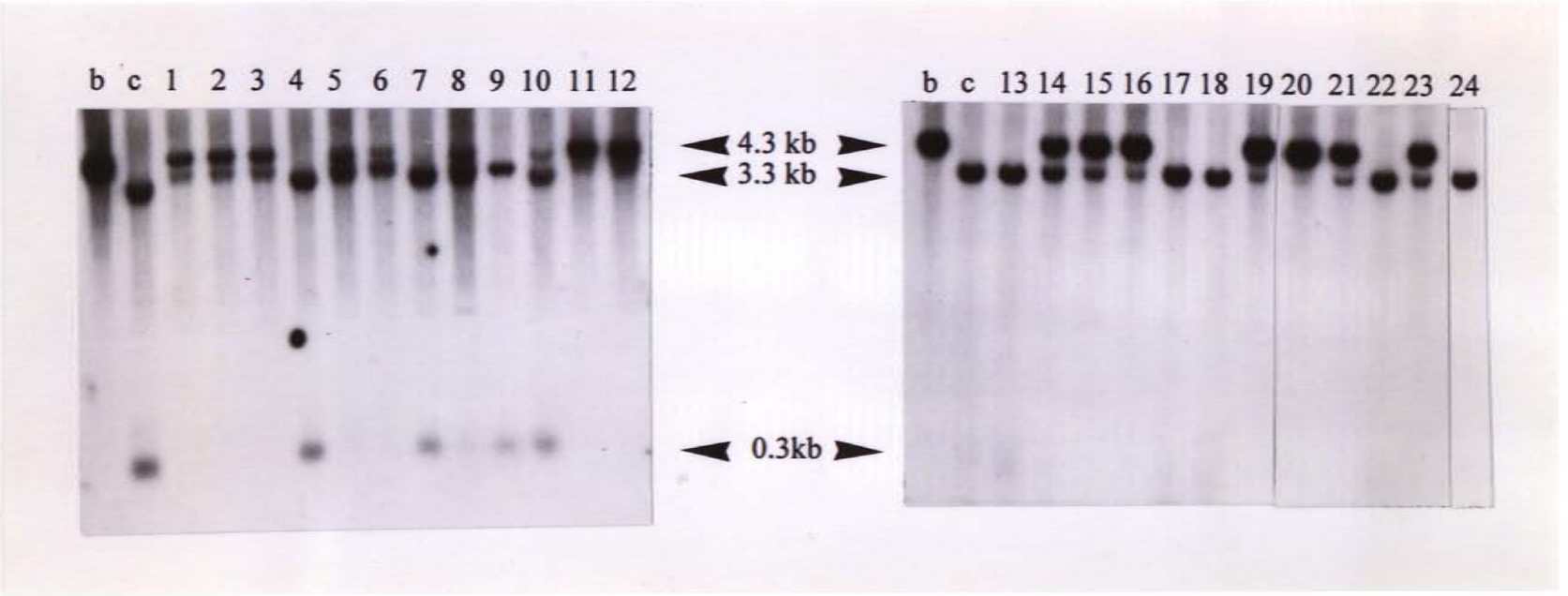


Fig. 2 (B) Southern blot analysis with the *p16* exon 1 probe for MM. For reference, lanes b and c are, respectively, normal blood DNA restricted with *EcoR*I alone (lane b, a 4.3 kb fragment simulates the fragment size seen as if the *Sac*II sites are methylated) and *EcoR*I plus *Sac*II (lane c, two smaller fragments 3.3 and 0.3 kb, represent unmethylation status). Lanes 1-24 are MM DNA all restricted with *EcoR*I and *Sac*II. Lanes 4, 7, 9, 13, 17, 18, 22, and 24 show no retention of the 4.3 kb methylation bands and hence unmethylated states. All others show mixtures of 4.3, 3.3 and 0.3 kb fragments and hence presence of aberrant methylation.

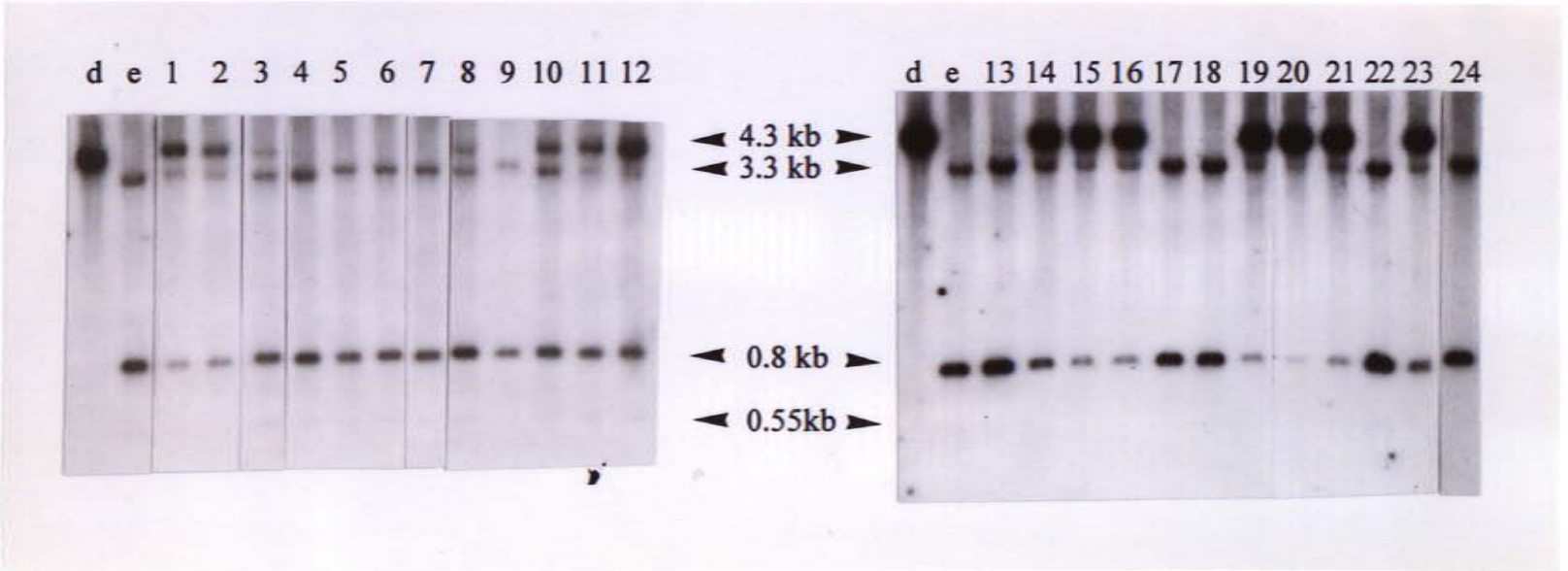


Fig. 2 (C) Southern blot analysis with the *p16* exon 1 probe for MM. For reference, lanes d and e are, respectively, normal blood DNA restricted with *EcoR*I alone (lane d, a 4.3 kb fragment simulates the fragment size seen as if the *Eag*I site is methylated) and *EcoR*I plus *Eag*I (lane e, three smaller fragments 3.3, 0.8 and 0.55 kb, two of which are of 0.8 and 0.55kb size, or 0.8 and 3.3kb if the 3' EagI site is methylated). Lanes 1-24 are MM DNA all restricted with *EcoR*I and *Eag*I. Lanes 4, 5, 6, 7, 9, 13, 17, 18, 22, and 24 show no retention of the 4.3 kb methylation bands and hence unmethylated states. All others show mixtures of 4.3, 3.3, 0.8 and 0.55 kb fragments and hence presence of aberrant methylation.

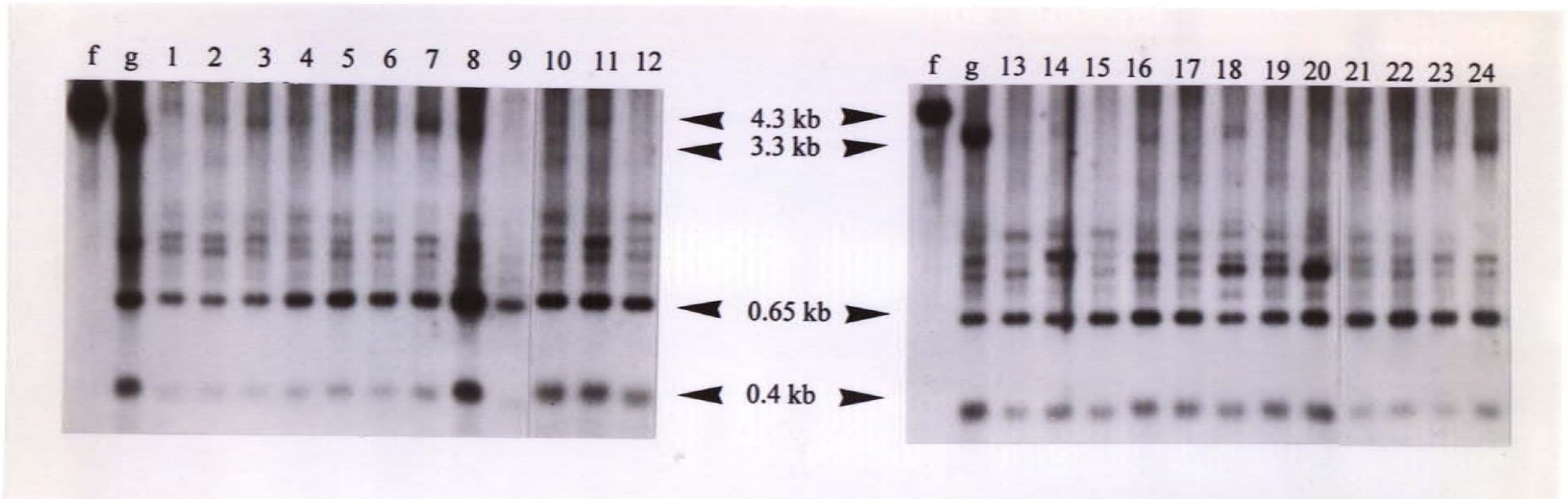


Fig. 2 (D) Southern blot analysis with the *p16* exon 1 probe for MM. For reference, lanes f and g are, respectively, normal blood DNA restricted with *EcoR*I alone (lane f, a 4.3 kb fragment simulates the fragment size seen as if the *Sma*I sites are methylated) and *EcoR*I plus *Sma*I (lane g, three smaller fragments, two of which are 0.4 and 0.65kb, or 0.65 and 3.3kb if the 3' *Sma*I site is methylated Lanes 1-24 are MM DNA all restricted with *EcoR*I and *Sma*I. All lanes show no retention of the 4.3 kb methylation band and hence unmethylated states on 5'CpG island region. Thr

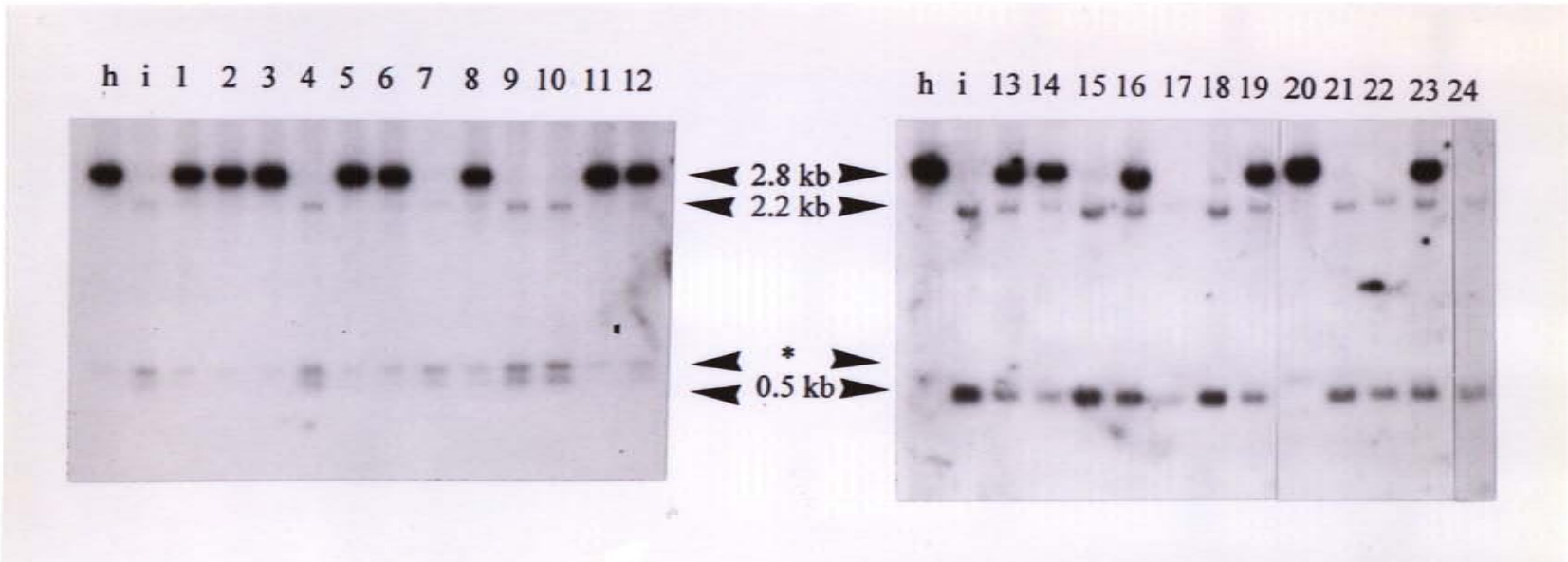


Fig. 2 (E) Southern blot analysis with the *p15* exon 1 probe for MM. For reference, lanes h and i are, respectively, normal blood DNA restricted with *Hind*III alone (lane h, a 2.8 kb fragment simulates the fragment size seen as if the *Eag*I site is methylated) and *Hind*III plus *Eag*I (lane i, two smaller fragments 2.2 and 0.5 kb, represent unmethylation status). * An unidentified fragment recognized by the *p15* exon 1 probe. Lanes 1-24 are MM DNA all restricted with *Hind*III and *Eag*I. Lanes 4, 7, 9, 10, 15, 17, 18, 21, 22 and 24 show no retention of the 2.8 kb methylation bands and hence unmethylated states. All others show mixtures of 2.8, 2.2 and 0.5 kb fragments and hence presence of aberrant methyaltion.

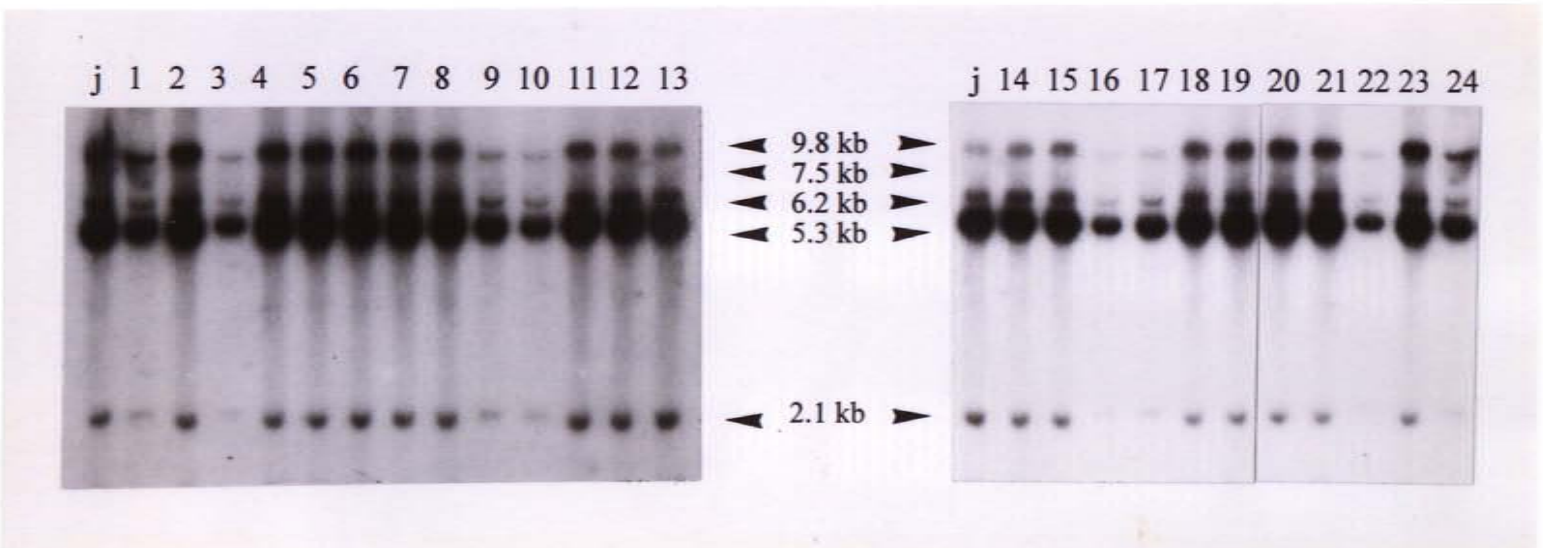


Fig. 2 (F) Southern blot analysis of Retinoblastoma (*Rb*) gene in 24 MM cases after *Hind*III digestion. The blot was successively hybridized with *Rb* cDNA probe (3.8 kb). For reference, lane j is placental DNA used as control. All cases show no homozygous deletions and gene rearrangements of *Rb*.

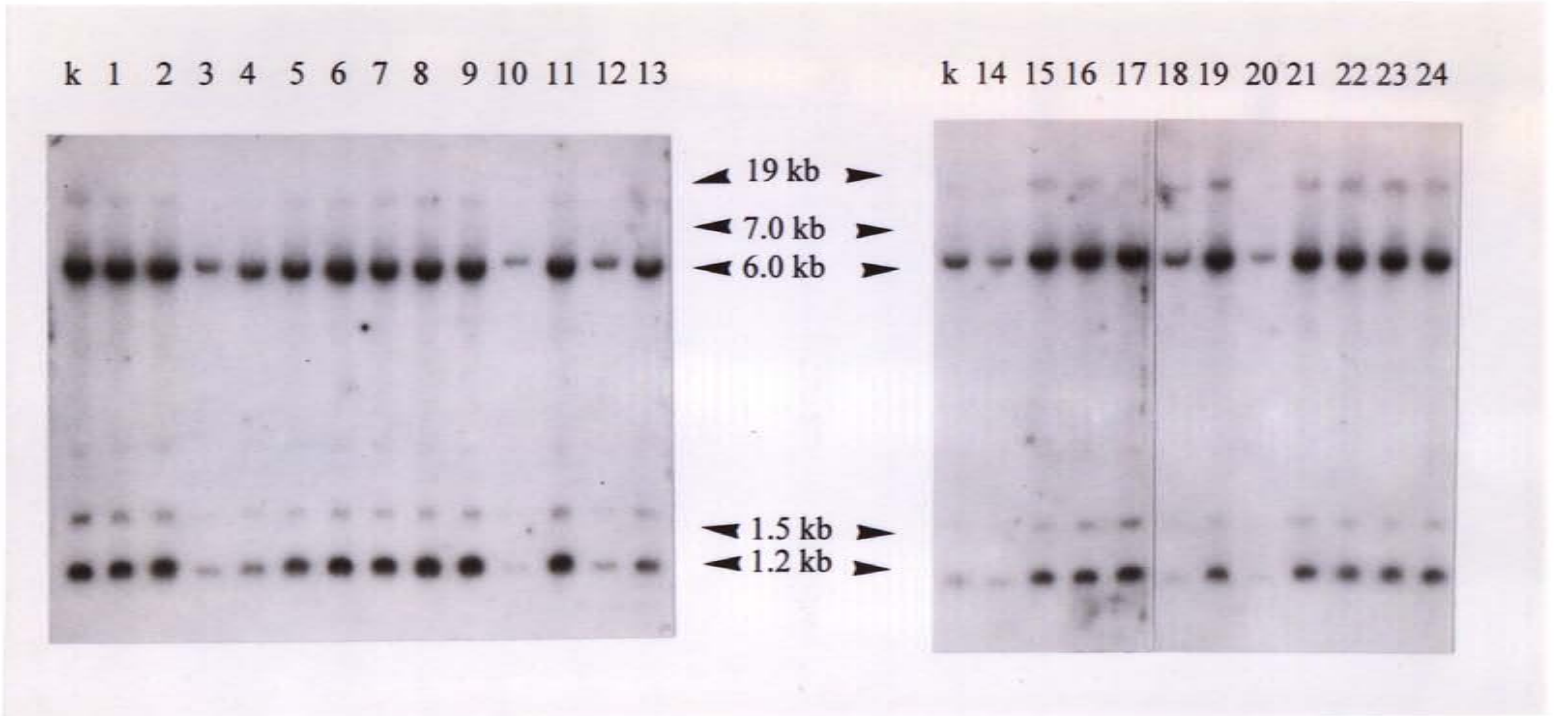


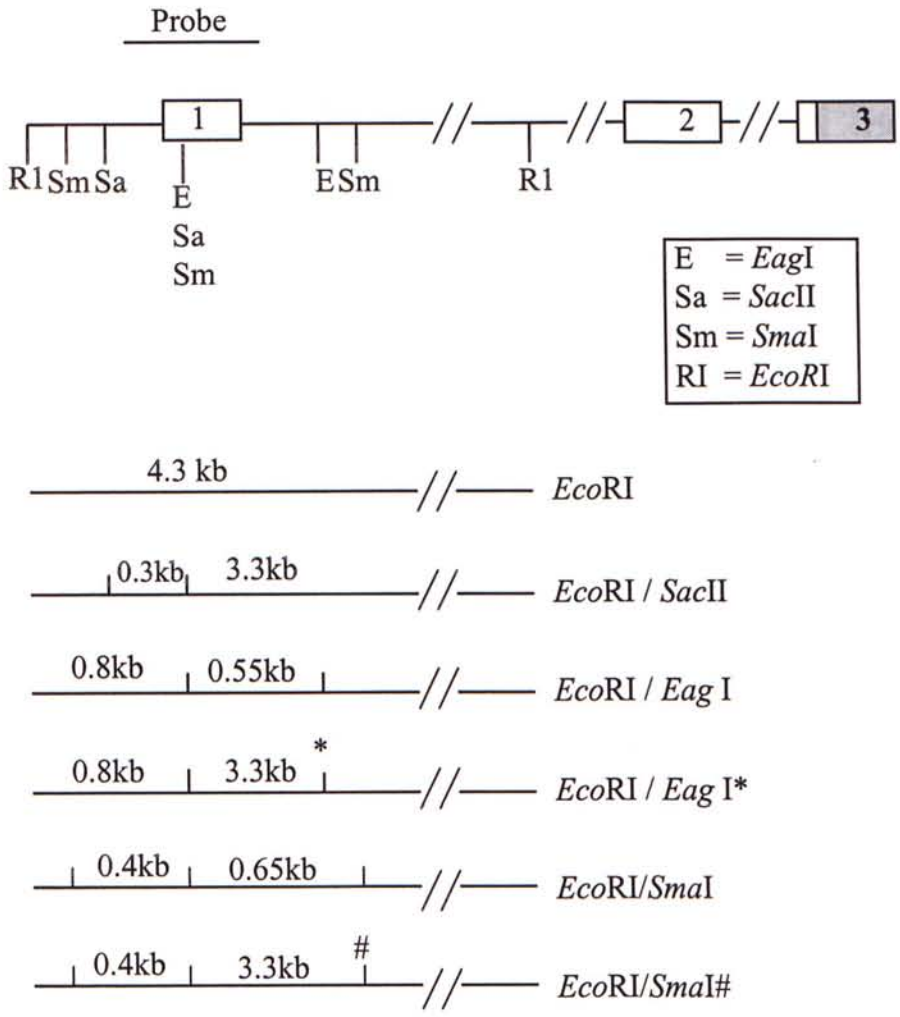
Fig. 2 (G) Southern blot analysis of Retinoblastoma (*Rb*) gene in 24 MM cases after *Hind*III digestion. The blot was successively hybridized with *Rb* cDNA probe (0.9 kb). For reference, lane k is placental DNA used as control. All cases show no homozygous deletions and gene rearrangements of *Rb*.

6. 2. 3 Hypermethylation of *p16*

The restriction map of *p16* is as shown in Figure 3A. With 5'CpG island hypermethylation of *p16*, the digestion by the methylation-sensitive enzymes would be protected and the 4.3 kb *EcoR*I flanking fragment would result. When the gene is unmethylated, digestion by the methylation-sensitive enzymes would yield smaller fragments. The expected sizes of these smaller fragments with different methylation-sensitive enzymes are depicted (Fig. 3A). The three methylation-sensitive enzymes (*Sac*II, *Eag*I or *Sma*I) have different methylation-sensitive restriction sites with respect to gene sequences and hence locations (Fig, 3A). In the twenty-four patients analyzed, eight patients (No. 4, 7, 9, 13, 17, 18, 22 and 24) were unmethylated at all *Sac*II, *Eag*I and *Sma*I sites of *p16* exon 1 (Table 6). They showed no methylation bands but two smaller fragments after digestion with *EcoR*I plus *Sac*II or *Eag*I or *Sma*I (Fig 2B, 2C & 2D respectively). Sixteen (67%) of our MM patients had hypermethylation at the *Sac*II site. They all showed retention of the 4.3 kb methylation bands together with the 3.3 and 0.3 kb bands arising from the normal or unmethylated cells after digestion with *EcoR*I and *Sac*II (Fig. 2B). With respect to the presence of tumor contents, the methylation band was prominent in twelve of them (No. 1-3, 11-12, 14-16, 19-21 and 23) and dominant in the other four (No. 5, 6, 8 and 10) (Fig. 2B). As referred from Table 6, it is noteworthy that two (No. 5 and 6) of the latter samples were not methylated at the *Eag*I site of *p16*. Moreover, none of our MM samples with repeated analyses, revealed methylation of the *Sma*I site. The methylation

density of the *p16* gene varies among our MM patients. According to Jones (1996), different methylation-sensitive restriction sites may be differentially methylated depending on the density and extensiveness of the methylation, which varies with the developmental stage of the specific tumor. Moreover, the level of transcriptional repression is dependent on methylation density. Partial methylation of CpG islands may result in down-regulation of the gene Involved. With progressive methylation, the gene may be totally inactivated.

Figure 3A

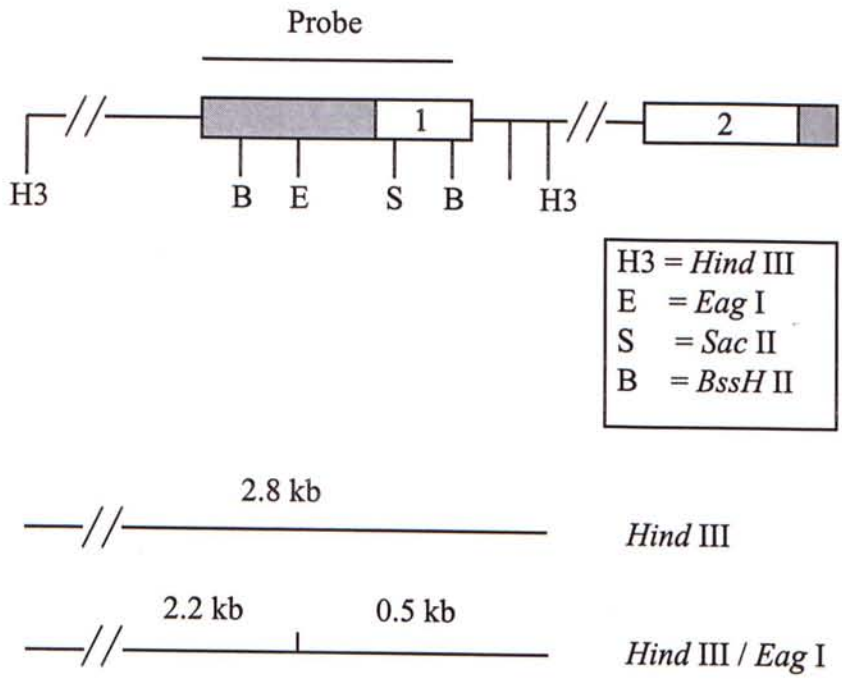


Restriction map of *p16*. Open boxes denote the three coding exons, the shaded box stands for the 3' untranslated region (UTR) of *p16*. Exon 1 resides in a 4.3 Kb *Eco*RI restriction fragment. Methylation-sensitive, rare base-cutting restriction enzymes include three *Sma*I (Sm), two *Sac*II (Sa), and two *Eag*I (E) sites. The location of PCR-generated probe used for Southern analysis is shown at the top. Below are the expected fragment sizes recognized by this probe when digested with the restriction enzymes shown in the normally unmethylated status. * The fragments produced by methylation of the 3' *Eag*I site seen in normal tissue. # The fragments produced by methylation of the 3' *Sma*I stie seen in normal blood. (Adapted from Merlo et al, 1995)

6. 2. 4 Hypermethylation of *p15*

Similarly, the restriction map of *p15* is as shown in Figure 3B. With no methylation, two smaller fragments of 2.2 and 0.5 kb would result from the digestion by *Hind*III and the methylation-sensitive enzyme *Eag*I. However, if there is 5'CpG island methylation of *p15* exon 1, a 2.8 kb restriction fragment would be produced. From the twenty-four patients studied, ten patients (No. 4, 7, 9,10, 15, 17, 18, 20, 21 and 24) were unmethylated. All showed no methylation bands but the 2.2 and 0.5 kb fragments (Fig. 2D). Fourteen patients (58%) had hypermethylation of *p15* (Table 6). All revealed retention of the 2.8 kb methylation bands with very strong signals and the 2.2 and 0.5 kb fragments. It thus appears that *p15* was completely methylated in all samples showing methylation.

Figure 3B



Restriction map of *p15*. The probe used for Southern analysis is shown at the top. Exons 1 and 2 are depicted with noncoding regions shaded. Methylation-sensitive restriction enzyme sites and *Hind*III sites are shown in the region including exon 1. The predicted sizes of restriction fragments used to analyze the methylation status of *p15* are shown at the bottom. (Adapted from Herman et al, reference 17)

6. 3 Hypermethylation of *p16/p15* and clinico-pathologic correlation

As shown in Table 7A, we found no homozygous deletions or gene rearrangements in any of our MM patients. However, high incidences of hypermethylation were detected in *p16* or *p15* (67% and 58% respectively) or concomitantly (54%). Three patients showed hypermethylation of *p16* (No. 10,15 and 20) alone while seven others were unmethylated for both genes (No. 4, 7, 9, 17, 18, 22 and 24). Hypermethylation of *p16* was found in 70% and 57% respectively among the pre-treated (n=17) and the post-treated (n=7) groups while that of *p15* occurred in 53% and 71% among the pre- and post-treated groups (Table 7B). In the seven patients having blastic morphology (No. 2, 6, 10, 11 15, 16 and 21), all (100%) showed methylation of *p16,* in contrast to the non-blastic group (n=17) in which *p16* was observed in 53% (p=0.026) (Table 7C). However, *p15* hypermethylaion in the blastic and non-blastic groups was observed in similar frequencies (57% Vs 53%). All the seven unmethylated cases revealed non-blastic morphology (Table 4). All three patients (100%) having plasmacytomas (No. 3, 5 and 11) were found to have concomitant hypermethylation of both *p16* and *p15* genes while this would only be seen in 48% of the non-plasmacytoma group (n=21)(Table 7D).

Table 7A *p16* and *p15* Status of MM patients

	Deletion (N=24)	Methylation (N=24)
p16	0%	67%
p15	0%	58%
Both	0%	54%
Either	0%	71%

Table 7B Methylation and Treatment Status of MM patients

Methylation	Pre-Tx (N=17)	Post-Tx (N=7)
p16	70%	57%
p15	53%	71%
Both	53%	57%
Either	70%	71%

Tx : Treatment

Table 7C Correlation of methylation of *p16/p15* and blastic Morphology

Methylation	Blastic (N=7)	Non-blastic (N=17)
*P16**	100%	53%
P15	57%	59%

* p=0.026

Table 7D Methylation and presence of plasmacytomas in MM

Methylation	Plasmacytoma (N=3)	Non-plasmacytoma (n=21)
p16	100%	62%
p15	100%	52%

Chapter 7

Discussion

7.1 Absence of homozygous deletions, gene rearrangements and mutations in *p16/p15* and *Rb*

No homozygous deletions or gene rearrangements were found by Southern blot analysis of *p16, p15* and *Rb* genes in the twenty-four MM patients studied. Using Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP), a sensitive method for the detection of point mutations in DNA fragments, no abnormal profiles were detected for *p16* (exon 1-3) and *p15* (exon 1-2) in all cases. Our findings were consistent with that in the literature. Quesnel et al studied *p16* gene on 18 and 30 patients with MM by Southern blot and PCR-SSCP analysis respectively, they detected no homozygous deletion on this gene and only two cases showed polymorphism. Hangaishi's group examined the status of *p16*, *p15* and *Rb* genes in 6 patients with MM. None of them showed homozygous deletions or mutations of these three tumor suppressor genes. Frequent deletions of *p16/p15* have been observed in lymphoid malignancies, particularly in pediatric ALL and B-lineage subtype (Okuda et al., 1995; Ogawa et al., 1995). However, thus far, no deletions or mutations of *p16* or *p15* have been found in MM despite its B-lineage origin (Ogawa et al., 1995; Quesnel et al., 1995; Stranks et al., 1995). Nevertheless, this may be consistent with the rarely observed loss of chromosome 9 or 9p in MM (Gould et al., 1988; Van den Berghe, 1989; Ng et al, in press, 1998). Previous cytogenetic findings showed that chromosomal gain of chromosome 9 was the predominant numeric changes in all the chromosomes studied. In all, monosomy 9 is the most rare (Zandecki et al, 1996). Recently,

our group also examined 18 Chinese MM patients by MGG/FISH using DNA centromeric probes specific for chromosomes 3, 7 and 9. We demonstrated high incidences of polysomies (83%) and trisomies (89%) 9. Monosomy 9 occurred only at 5.6% (Ng et al, in press, 1998). We may suggest that deletions of *p16/p15* are rare.

Residual *p16*, *p15* or *Rb* signals in tumors contaminated by normal cells may pose technical problems in the detection of homozygous deletions of the genes. In general, the reliability of deletion detection drops with increased number of normal cells and hemizygous deletions are difficult to determine and differentiate from non-deleted cases when normal contaminants are more than 30%. In our patient samples, 42% (10/24) of the cases had more than 70% of the tumor cells. No homozygous deletions were observed, although it is possible that it might be an underestimation. In this study we only focused on the detection of homozygous deletions of *p16*, *p15* and *Rb* genes. The incidence of hemizygous deletions of these genes was not evaluated. One of the reasons was that in our experience with Southern blot analysis, demonstrating of loss of only one copy of a gene in tumor cells contaminated by up to 20% or over 30% of normal residual cells is difficult. Furthermore, as for the tumor suppressor genes (see section on Knudson's Model), inactivation presumably requires the inactivation of both alleles, and the relevance of hemizygous deletions to the oncogenic process may be more hypothetical.

7. 2 Hypermethylation of *p16/p15*-an alternative way for gene inactivation

Our study is the first demonstration that alterations of *p16* and *p15* occurred at high incidences in multiple myeloma, not by homozygous deletions or mutations, but solely by hypermethylation of 5'CpG islands which may be a specific mechanism in this disease. This methylation pattern was not found in normal blood and normal marrow controls. Such high frequencies of involvement of these genes in MM make them hitherto the most common genetic abnormalities in this disease. Thus, these alterations may play an important role in the pathogenesis of MM.

In our group of relatively high stage MM patients, high frequencies of involvement of either gene or concomitant were observed. However, since hypermethylation of *p16 / p15* was found in both low and high stage patients and in similar incidences in pre- and post-treated groups, it may suggest that they are early events in MM and their role in tumor initiation can be speculated. In line with this speculation is the evidence from studies with immortal but not tumorigenic mouse cell lines, supporting the idea that methylation changes occur very early in the transformation process (Jones,1996). The fact that concomitant hypermethylation of both *p16* and *p15* happened in all three cases of MM with plasmacytomas may underscore their pathogenetic significance in their development. Furthermore, the finding that all seven MM patients with blastic morphology revealed *p16* hypermethylation ($p=0.026$) while all seven unmethylated cases had non-blastic morphology indicates that *p16*

hypermethylation may be associated with blastic disease. This is further supported by the absence of plasmacytoma and blastic morphology in all the unmethylated cases. In three cases, only the *p16* gene was involved. This may suggest that *p16* was the target gene in the pathogenesis in these cases. In one case, only the *p15* gene was methylated which indicated that *p15* may be the target for inactivation in the tumorigenesis in this particular case.

7.2.1 Methylation of *p15* gene

Unlike solid tumors, the loss of *p15* gene function is a rather dominant event in tumors derived from the hematopoietic cell system, and CpG island hypermethylation is by far the dominant mechanism involved. This is most dramatically apparent from large studies of patients with both the adult and childhood types of acute myelogenous leukemia (AML), in which 85% and 65%, respectively, of the non-cultured tumors have this change in the *p15* gene (Herman et al, 1996; Herman et al, 1997). The methylation pattern seen for AML also predominates in adult ALL (71%) and pediatric B-cell ALL (48%) (Herman et al., 1996). No homozygous deletions or mutations of either the *p15* or *p16* genes have been found in these studies, and the *p16* gene was not simultaneously hypermethylated (Herman et al., 1996; Herman et al., 1997).

Concomitant hypermethylation, uncommon thus so far in the literature of the study of *p16* and *p15* with the exception of Burkitt's lymphoma (50%), is a rather common phenomenon in our patient group. Both *p16* and *p15* are

CDK4/6 inhibitors, which inhibit their interaction with D1 cyclins and thus preventing the phosphorylation of the pRb and arresting the cell at G_1 phase. *p15* is up-regulated by transforming growth factor-β (TGF-β) which plays and important role in growth suppression of hematopoietic precursor cells in the bone marrow where most of the myeloma cells would be accumulated. This growth inhibitory activity of TGF-β has been shown actually effected through this *p15* regulation. In addition to *p16*, the inactivation of *p15*, which may be particularly important in bone marrow environment where there is increased TGF-β activity, would thus be critical in exerting the major up-stream inhibitory control of *Rb* function. This may also explain the need for concomitant hypermethylation of both *p16* and *p15* in MM. The consequent loss of cell cycle arrest and increased cellular proliferation would be further enhanced by the release of IL-6.

7.2.2 Methylation of 5'-CpG island of *p16/p15* and lack of gene expression

Other investigators have demonstrated that hypermethylation of 5'CpG islands of *p16/p15* can lead to transcriptional silencing proven by the absence of transcripts (Merlo et al, 1994; Herman et al, 1995). *In vitro* experiments have also demonstrated the reversible nature of this transcriptional block by the introduction of demethylation agent, 5-aza-2'-deoxycytidine, which leads to re-expression of these genes. Aberrant methylation of *p16* and *p15* may be analogous to homozygous deletion, leading to loss of *p16* and *p15* expression respectively and a selective growth advantage to tumor cells. The association of hypermethylation and inactivation of these genes in MM has recently been

demonstrated by our group. We investigated methylation status and transcription of these genes in six MM cell lines by Southern blot analysis and RT-PCR (Wong et al, in press, 1998). Aberrant methylation of *p16* was found in all six MM cell lines studied. However, loss of *p16* transcription was demonstrated only in four with extensive methylation in the 5' CpG islands. Conversely, only one MM cell line showed extensive methylation of *p15,* which was associated with *p15* transcriptional block. Restoration of gene transcription was observed after treatment of the demethylating agent 5-aza-2'-deoxycytidine (Wong et al, in press, 1998). The above data show that the *p16* and *p15* genes are silenced in MM by hypermethylation of the 5' critical region and the loss of the gene transcripts can be reversible, consistent with findings in other tumors in the literature.

7.2.3 Comparison of methylation status of primary samples and cell lines in MM

Comparing the results from our primary MM samples and the cell lines, *p16* hypermethylation occurred at high frequency (67% in primary samples and 100% in cell lines), whereas *p15* hypermethylation was shown in only one of the MM cell lines (16%), but at a higher percentage (56%) in primary MM. This discrepancy may be due to the localization of plasma cells in bone marrow environment in the primary MM as suggested by the authors (Wong et al., in press, 1998). The primary MM specimens were all obtained from the bone marrow, where accumulated TGF-β would stimulate *p15* expression and hence suppress tumor growth. It is possible that TGF-β may impose a selective

pressure upon inactivation of *p15* expression in the bone marrow environment (Herman et al, 1996). However, MM cell lines were derived from different sources, including peripheral blood, bone marrow aspirates and pleural diffusions. It is also possible that cell lines may loss their methylation status after long period of *in vitro* cell line culture since in our *in vitro* culture system there is no TGF-β as supplement.

In the cell line studies, transcriptional silencing of the *p16* gene was only observed in four of the six MM cell lines where the 5' CpG islands of *p16* was densely methylated. In contrast, *p16* transcription was detected in the other two MM cell lines, which had *p16* alleles with unmethylated sites as well as partially methylated sites. The cell line data support that the density of methylation in *p16* gene correlates with the level of gene transcription in MM. Unlike the cell line data, our primary samples showed no partial methylation patterns. Thus, this may suggest the complete loss of gene transcription of these genes in our primary MM cases.

7.2.4 Progressive gene inactivation by random methylation errors

For the progressive inactivation of tumor suppressor genes by abnormal methylation of CpG islands, tumor suppressor genes contain CpG islands which, in common with other autosomal genes, are not normally methylated. Random methylation errors of these CpG sites leads to reduced gene expression, resulting in the clonal selection of cells with these heritable epigenetic defects.

Further methylation can result in the eventual paralysis of the gene by herochromatinization, giving rise to further selection of cells with methylation defects. Finally, focal hypermethylation of CpG islands on gene regulatory region occurs and leads to complete silencing the gene. Although we do not presently understand whether *de novo* methylation of 5'CpG islands represents a random epigenetic alteration or occurs in a regulated manner, it seems likely that once methylation is established in the 5' regulatory region of a tumor suppressor gene, clonal selection will ensue leading to tumor progression.

7.2.5 The lack of correlation of tumor contents revealed by the Southern blot analysis and morphologic assessment

The problem of detection of specific gene deletions in primary tumor samples with normal cell contamination has been addressed previously. The examination of methylation of the 5'CpG islands of *p16/p15* would be much less affected by the presence of normal cells. This is because they would remain unmethylated and retention of a specific DNA fragment could only happen in hypermethylated genes from the tumor cells. However, the assessment of tumor infiltration on morphological ground has potential limitation by sampling variation that may lead to a lack of correlation with the tumor contents revealed by the Southern analysis. This fact is particularly important in MM as the disease itself tends to develop focally. Also, because gain in copy numbers of chromosome 9 is a predominant phenomenon in MM by both cytogenetic and FISH studies, and the methylation status of the additional copies of chromosome 9 is variable, there is a variable relationship between tumor cell numbers and the

chromosomal 9 DNA contents in the MM patients. As demonstrated by our FISH study on MM cells, the variability of the percentages of cells with different polysomies of the same and different chromosomes suggested the presence of heterogeneous populations of MM cells (Ng et al, in press, 1998). This heterogeneity may similarly exist in terms of the methylation status among the MM cells, which may further account for the lack of correlation between tumor contents revealed by the intensities of methylation signals and the morphologic evaluation. These various factors probably explain the discrepancies of tumor contents seen in some cases (e.g. Nos. 5, 12, 14 and 15). While morphological assessment gave them fairly low tumor percentages (25%-36%, Table 5), methylation occurred with the majority (>50%) of DNA. In these four cases, the morphological evaluation may probably under-estimate the tumor presence or the chromosome 9 DNA contents.

7.3 Knudson's two-hit model of tumorigenesis

According to Knudson's two-hit model of tumorigenesis, both copies of targeted tumor suppressor genes have to be inactivated. Allelic inactivation could be due to deletions, mutations or hypermethylation and a combination of these (e.g. homozygous deletion, hemizygous deletion and mutation or hypermethylation, etc.) occurring in both copies of a gene can finally lead to gene inactivation. Although frequent deletions of *Rb* (50-70%) have been identified in MM, a majority are hemizygous and not associated with lack of p*Rb*

protein expression. Since bi-allelic loss of *Rb* and hence inactivation only occurs in 10% of MM (Juge-Morineau et al, 1995), this may suggest that *Rb* is not the major target gene in MM. Dysregulation of Rb function resulting from upstream control abnormalities can be speculated, and in fact, supported by our finding of a high frequency of (71%) *p16/p15* hypermethylation (transcriptional repression or inactivation).

7.4 Inverse relationship of *p16* and *Rb*

Complementary distribution of inactivation of both *p16* and *Rb* genes have been quite well known. Only either of the two was inactivated in various tumors (Okamoto et al, 1994; Aagaard et al, 1995; Yeager et al, 1995; Shapiro et al, 1995; Parry et al, 1995). If the genes involved in the up-stream control of *Rb* pathway have been inactivated, further inactivation of *Rb* may become redundant and confer no added growth advantage for clonal selection. Examples are best seen in lung carcinomas. While small cell lung cancers with frequent *Rb* inactivation rarely show *p16* involvement, non-small cell lung cancers with rare *Rb* inactivation are more often found to have *p16* inactivation (Merlo et al, 1995; Otterson et al, 1994). Our finding of inversely related alterations of *Rb* and *p16/p15* in our primary MM cases further supports this notion. While hypermethylation (hence inactivation) of *p16/p15* was found at high incidences, by Southern blot analysis, no homozygous deletions or gene rearrangements were observed on *Rb*.

Inactivation of both *p16* and *Rb* genes had not been described in hematological malignancies until lately when Hangaishi et al (Hangaishi et al, 1996) reported four cases of primary lymphoid malignancies affected by simultaneous presence of homozygous deletion of *p16* and loss of expression of pRb. This rare phenomenon may be explained on the other hand, as proposed by the authors, from several lines of evidences, which suggest that functional *Rb* protein is essential for cell cycle inhibition by *p16* (Guan et al, 1994; Koh et al, 1995; Lukas et al, 1995). *p16* inactivation in a cell without functional *Rb* protein is not likely to confer an additional growth advantage upon the cell any more. In these double mutants, deletions might precede *Rb* inactivation, the latter could then contribute to advantageous tumor growth hereafter from a total cancellation of all the inhibitory effects of the other CDKs via *Rb* protein. The involvement of *p16/p15* and *Rb* by Southern blot analysis and pRb expression were also studied on six cases of MM. No homozygous deletions in *p16/p15* or *Rb* or loss of pRb expression were observed. However, methylation status of *p16/p15* in these MM cases was not evaluated.

In the same study on primary lymphoid malignancies, they observed that inactivation of the individual tumor-suppressor genes (MTS1/*p16*, MTS2/*p15*, *p53*, and *Rb*) seemed to occur independently to each other except for the *p16* and *p15* genes. In addition, they suggested that disruptions of multiple tumor suppressors in a tumor cell confer an additional growth advantage on the tumor. This proposal may be in line with another school of thought, which supports that gene dosage effect might also be critically important for its tumor suppressor

function. Perhaps inactivation of a single allele may lead to a functional effect secondary to decreased expression due to haplo-insufficiency since the loss of one copy of the gene could partially abrogate its intrinsic functions.

7. 5 Implications of our findings

There are two implications for MM from our findings. 1. Since hypermethylation of *p16/p15* is a common event in MM, most patients can be benefited from this investigation. With Methylation-specific PCR technique as described by Baylin et al, the methylation status can serve as a tumor marker for the detection of minimal residual disease. More importantly, it may be used to check for tumor contamination in autologous bone marrow or peripheral stem cell harvests in autologous bone marrow transplantation. 2. It throws light on therapeutic innovations of MM treatment. Up till now, MM is still a uniformly fatal disease with chemotherapy. Use of demethylating agents either *in vitro* as a pretreatment of the autologous graft before transplantation or *in vivo* as a therapeutic agent incorporated into drug developments or via gene therapy by targeted delivery may be speculative at this stage but effective in future.

7.6 Future studies

1. To pursue on the studies as implicated in the last section on MM patients during autologous bone marrow transplantation or by longitudinal

investigation for the establishment of methylation status as a tumor marker for the evaluation of tumor contamination, minimal residual disease and patient's progress from treatment.

2. Furthermore, if *Rb* is not the target gene as supported by our data above, another possibility of frequent hemizygous *Rb* deletion is due to the co-deletion of an unidentified tumor suppressor gene residing on the 13q14 locus, which may be another target gene for MM. Similar observations of absence of *Rb* inactivation have been made in some other solid tumors with frequent loss of 13q region where alternative target genes have been suggested (Maestro et al, 1996; Cooney et al, 1996). Investigation of the loss of heterozygosity (LOH) using an *Rb* sequence polymorphism and polymorphic microsatellite markers surrounding the *Rb* gene on the long arm of chromosome 13 could be conducted for further mapping of the deleted region on chromosome 13q and localization of the potential alternative tumor suppressor gene other than *Rb.*

3. Finally, to provide functional evidence in support of the hypothesis that *p16/p15* genes are functional growth suppressor genes in MM, we have to assess the growth suppressive function of these genes by gene transfer study. The effects of the introduction of full-length *p16/p15* cDNAs into inactive *p16/p15* MM cell lines as compared to MM cell line with endogenous wild type *p16/p15* alleles could be examined. Marked growth suppression in the former as in contrast to the latter may be expected.

1. D1. Blood 88:268, 1996

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